Cardiovascular risk and its determinants in high risk patients

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CARDIOVASCULAR RISK AND ITS DETERMINANTS

IN HIGH RISK PATIENTS

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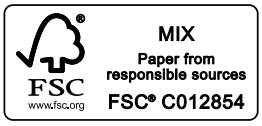

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CONTENTS

CHAPTER 1

INTRODUCTION AND OBJECTIVES

Advanced glycation endproducts: formation pathways

Glucose and non-glucose dependent pathway

With increasing glucose levels, various biochemical processes will change or will be stimulated or activated, and other endproducts will be formed than in normoglycemic conditions. For example, the prostaglandins and leukotrienes formed during hyperglycemia are different from those formed during normoglycemia. Also, various types of leukocytes are stimulated to form enzymes contributing to glycation. Still, increased glucose levels per se are eliciting only a small part of the possible problems and complications, associated with diseases like diabetes mellitus (DM). In patients with hyperglycemia, glucose will – quite often irreversibly – bind to other molecules inside the human body like aminoacids, and to a lesser extent fatty acids and nucleic acids, leading to glycation of these molecules. Glycation can be accompanied by oxidation, contributing to further adverse changes (see also below). The basic chemistry of the formation of advanced glycation endproducts (AGEs) was first described by the French food chemist Louis Camille Maillard in 1912. The so-called Maillard reaction is based on a non-enzymatic reaction between amino acids and glucose (Figure 1), and is the classical pathway of AGE formation.

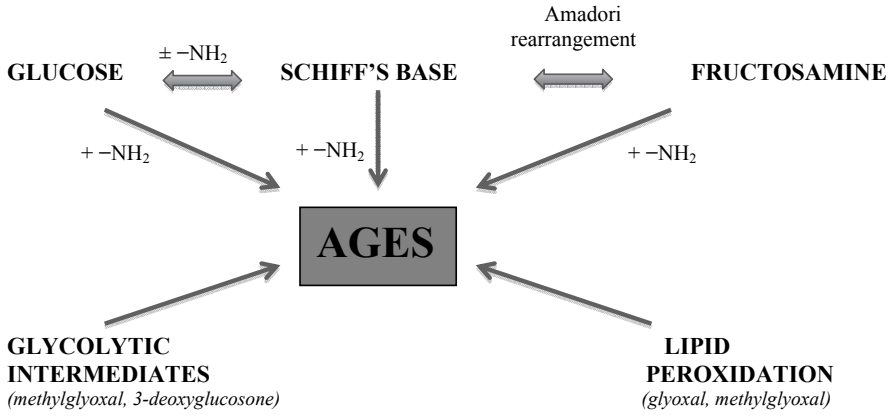

Figure 1. Schematic view of the complex Maillard reaction and other pathways leading to the formation of AGEs.

The starting point of the Maillard reaction is the formation of a Schiff base: an aldehyde group of a glucose molecule combines with an amino group of an amino acid molecule in a protein to form an imine or Schiff base. The next step is the formation of an Amadori product, an organic reaction; this re-arrangement of the Schiff base means the move of the hydrogen atom from the hydroxyl group adjacent to the carbon-nitrogen, leaving a ketone. And the last step is the oxidation of the Amadori products, most often by transition metal catalysis, which leads to the irreversible formation of AGEs. This endogenous and slow formation of AGEs is concentration-dependent at the early stage of the Maillard reaction and accelerates under circumstances of hyperglycemia (1,2).
A non-glucose-dependent AGE pathway involves the inflammatory stimulation of neutrophils, monocytes, and macrophages, which produce myeloperoxidase and NADPH oxidase enzymes which are able to form reactive carbonyls and Nε-carboxymethyllysine (CML) by oxidizing amino acids (3). This could explain the production of increased AGE formation in various inflammatory diseases, such as systemic lupus erythematosus and rheumatoid arthritis (4-7).

Oxidative stress
Besides the classical Maillard reaction, oxidative stress is another pathway that results in AGE-formation. Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the protective mechanisms against oxidative stress (see below). Mitochondrial overproduction of ROS is a consequence of intracellular hyperglycemia or increased oxidation of fatty acids and plays a central role in the formation of intracellular methylglyoxal (MGO)-derived AGEs. Oxidation of fatty acids, resulting in part from pathway-specific insulin resistance, and non-oxidative mechanisms (e.g. anaerobic glycolysis) are responsible for the formation of dicarbonyls, which in turn bind amino acids to form AGEs. This so-called dicarbonyl stress pathway encompasses the rapid formation of reactive intermediate products such as MGO and 3-deoxyglucosone, which are also termed dicarbonyls, oxoaldehydes or reactive carbonyl compounds (8-10). Lipid peroxidation in the presence of ribonuclease A, a protein that contains neither enzymatically nor nonenzymatically attached carbohydrates, results directly into the formation of AGEs, such as CML (11).

Patients with end stage renal disease (ESRD) on hemodialysis have much higher AGE levels than healthy subjects because of increased levels of oxidative stress and reduced antioxidant levels. Furthermore, in uremia CML and pentosidine production is supposed to be determined both by an increased level of oxidative stress and the availability of precursors of these AGEs, irrespective of the presence or absence of a hyperglycemic state (12-14).

Exogenous sources of AGEs

Besides the above mentioned endogenous sources of AGEs, dietary intake and smoking are the prominent exogenous sources of AGEs. The oral bioavailability of AGEs absorbed from ingested food is estimated at about 10% and these diet-derived AGEs are similar to native AGEs with respect to the prooxidant and proinflammatory characteristics (15,16). Auto-oxidation of glucose is accompanied by the generation of superoxide radicals and ingested glycoxidation and lipoxidation products can accelerate free radical generation and oxidative and carbonyl stress (17). Cigarette smoke contains high concentrations of glyoxal and MGO due to the thermal decomposition of saccharides, which are the most likely mediating factors of smoking associated AGE formation (18).

Removal of AGEs

There are a few key factors playing an important role in the removal of AGEs. An important first step is the degradation of AGE linked proteins to AGE-peptides by macrophages (19). Subsequently, adequate renal clearance capacity is necessary for the effective removal of these fractions of the AGEs. In renal failure, there is a profound decrease in clearance of AGE-free adducts (20,21). As a consequence, decreased renal clearance of AGEs contributes to the endogenous accumulation of AGEs.

Besides the kidney, the liver may also be involved in the removal of plasma AGEs. Animal studies and in vitro experiments have suggested an active role of the liver in AGE metabolism by showing hepatic uptake by liver endothelial cells and by Kupffer

cells. Sinusoidal liver cells were found to play a role in the removal of AGEs (22,23). Markedly elevated levels of serum AGEs have been found in patients with liver cirrhosis, which correlated with the severity of the disease and ameliorated by liver transplantation (24). To summarize, both kidney and liver failure can contribute to the accumulation of serum AGEs.

Pathogenetic role of AGEs

An important effect of AGEs is the crosslinking with proteins, nucleic acids and lipids, resulting in structural changes, malfunction and reduced breakdown. These cross-links and accumulation on long-lived proteins, such as skin collagen or in the vascular basement membrane, affect the structure and function of the vascular wall resulting in vascular damage. Tissue accumulation of AGEs is a long-term process. Quantitation of the collagen-bound AGEs (in the skin) could reflect 'metabolic memory' over the past 15 years, because the lifetime of skin collagen has been estimated to be 15-20 years (25). Another effect of AGEs is the adherence to cellular binding sites, resulting in depletion of cellular antioxidant defense mechanisms such as vitamin C and glutathione and the generation of oxygen free radicals (26,27). Vitamin C and glutathione are examples of the non-enzymatic antioxidant system and glutathione peroxidase, superoxide dismutase, catalase and the peroxiredoxin enzyme family are examples of the enzymatic antioxidant system. They are all part of the antioxidant defense system present in all aerobic organisms. Exhaustion of the antioxidant protective system as well as mitochondrial glycation, may enhance oxidative stress, introducing a vicious circle. Finally, AGEs can bind to cell membrane receptors which may have positive or negative effects. AGE receptors or scavenger receptors that enhance clearance of AGEs are part of the human immune defense system, which is a protection against the vascular damaging effects of AGEs. The best known pro-inflammatory receptor for AGEs is called RAGE, a representative AGE receptor on endothelial cells and part of the immunoglobulin superfamily (28,29). Binding of AGE to RAGE could result in activation of intracellular pathways e.g. activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and the subsequent expression of endothelial adhesion molecules and tissue factor. These factors,

including the release of cytokines, finally contribute to endothelial dysfunction and other deleterious vascular effects (3,30-33).

Pathogenetic role of AGEs in diabetes mellitus

Several different mechanisms concerning specific AGEs seem to play a pathogenetic role in the development of microvascular complications in DM. Cross-sectional studies have shown the association of increased AGE levels and diabetic complications. Skin collagen bound AGEs such as CML and pentosidine have been shown to have predictive value for microvascular complications, independently of glucose levels or recent HbA1c values (34). About 25 years after diagnosis of DM, (at least) background diabetic retinopathy will have developed in the majority of diabetic patients. CML and pentosidine skin collagen accumulation is associated with the severity of retinopathy in type 1 DM, independently of age and diabetes duration (2). Several studies have shown evidence for increased CML reactivity in diabetic retinas (35-37). Glycation of lens crystalline and subsequent oxidation play a role in accelerated cataract formation in DM (38). Increased levels of pentosidine were found in the vitreous body of patients with diabetic retinopathy when compared to a nondiabetic control group (39). The importance of the role of AGEs in the development of diabetic retinopathy has also found evidence in animal studies with the AGE inhibitor aminoguanidine. Aminoguanidine prevented microaneurysm formation, pericyte loss, and the development of accelerated diabetic retinopathy (36).

AGEs also play an important role in the development of diabetic nephropathy, which develops in approximately 40% of patients with DM. Increased levels of CML, pyrraline and pentosidine have been found in kidneys of diabetic patients with or without ESRD and circulating AGE-peptides correlates with the severity of renal function impairment (40,41). Experimental studies have shown the role of increased oxidative stress and overexpression of RAGE resulting in histological and functional changes contributing to diabetic nephropathy (41,42). Moreover, pharmacological inhibition of AGE-formation by aminoguanidine prevented the development of kidney lesions, albuminuria and mesangial expansion in diabetic rats (43,44).

The third microvascular complication is diabetic neuropathy, which eventually will occur in the large majority of patients with DM (80-90%). Several mechanisms seem

to play a pathogenetic role concerning AGEs and neuropathy: accumulation of AGEs in vasa nervorum resulting in wall thickening, occlusion and ischemia of nerves, and myelin damage with segmental demyelinisation. There is also glycation of proteins of the axonal cytoskelet and nerve fiber regeneration may be reduced because of glycation of the nerve growth factor and other growth factors (45).
Each microvascular complication of DM has its own pathogenetic mechanism with AGE involvement resulting in microvascular damage.

AGEs and atherosclerosis
AGEs have been localised in atherosclerotic lesions, fatty streaks, lipid containing smooth muscle cells and macrophages in patients with DM, with a correlation between tissue AGE concentration and the severity of atherosclerotic lesions (56-59). AGEs act directly on the arterial vessel wall by inducing cross-links on long-lived proteins such as collagen, which alters vascular structure and function and promotes vascular stiffness. Multiple potential mechanisms might be responsible for AGE induced enhanced atherosclerosis. AGEs capture nitric oxide and impair LDL removal by trapping LDL in the subendothelium and decreasing LDL receptor recognition of AGE-modified LDL (60). AGEs linked to apolipoprotein B impairs its hepatic clearance, and induces retention of LDL in the arterial vessel wall with an increased production of foam cells. This in turn accelerates atheromatous plaque formation (61). AGEs also upregulate the vascular cell adhesion molecule-1 (VCAM-1) expression by activating the key nuclear transcription factor NF-κB (62). Indirect evidence about the deleterious effects of AGEs was found in animal studies, using pharmacological interventions e.g. AGE cross-link breaker aminoguanidine and using sRAGE resulting in reduced accumulation of AGEs and suppression of vascular damage (63,64).

Pathogenetic role of AGEs in renal failure and ESRD
In patients with chronic renal failure and ESRD, irrespective of having DM or not, the reactive carbonyl compounds and CML levels in plasma as well as in the skin are markedly increased (46-49). Several mechanisms are supposed to be responsible for these increased serum AGE levels: impaired clearance of AGEs and AGE precursors, as well as increased levels of oxidative stress with the development of reactive oxygen

species. Interestingly, chronic uremia is supposed to be a state of increased oxidative stress with an accelerated production of CML and pentosidine even in the absence of glucose (50-52). Miyata postulated the existence of factors either producing unknown precursors or catalysing the formation of AGEs under uremic conditions. He also suggested that AGE accumulation including pentosidine and CML is linked to a redox imbalance in these conditions (13). Additionally, uremic toxins result in higher levels of oxidative stress, which is also facilitated by the dialysis membrane in hemodialysis, and activate polymorphonuclear leukocytes (53-55). All these mechanisms will lead to increased serum AGE levels, finally resulting in an accelerated accumulation of tissue AGEs which in turn contributes to vascular damage.

Assessment of AGEs

It is a challenge to determine the best and most proper assessment of the level of tissue AGE accumulation, because the structure of AGEs is complex and heterogeneous, and each technique has its own limitations.

Serum AGEs

Different assays concerning the measurement of serum AGE levels have been developed, but these assays are only applicable to AGEs with known biochemical structures. These assays do not represent the whole group of serum AGEs. For example, the immunoassay method, using different antibodies against different AGEs in an ELISA is not very laborious, is cheap and feasible for clinical use, but reproducibility and sensitivity still remains a problem (65,66). On the other hand, liquid chromatography – mass spectrometry (LC-MS) is a more specific and reliable method for measuring serum AGE levels, but this technique is expensive and time-consuming.

All of the techniques of serum AGE level measurement do not accurately reflect the accumulation of tissue AGEs (67-72). Turnover of AGE-linked proteins in serum is much higher and is more dependent on renal clearance than the AGE crosslinked on long living proteins like collagen or elastin. Therefore, the correlation of serum

AGEs with tissue AGE accumulation is unclear. Moreover, the reproducibility of many (immune) assays between different laboratories is also rather poor.

Tissue AGEs

Assessment of AGEs in skin biopsies comprises invasive and elaborate methods: extensive preparation of samples, high-performance liquid chromatography (HPLC), LC-MS or tandem MS. It also includes the determination of collagen linked fluorescence and the protein cross-linking index as markers of tissue AGEs. The latter two methods both provide an indirect quantitative measure of tissue AGE accumulation. The fluorescence method has a proven association with (diabetic) complications (35,73). The disadvantages of this method are the interference with non-AGE fluorophores and there is no detection of non-fluorescent AGEs such as CML and pyrraline. The method of collagen crosslinking has a low specificity, is only valid to collagen rich tissue, and its reproducibility is unclear. Because of the invasive character of both methods, they are not feasible for AGE monitoring.

Tissue AGEs can also be measured by the skin autofluorescence reader, an easily applicable, and noninvasive instrument, the use of which is not time consuming. Skin autofluorescence (AF) has been validated against AGE measurements in dermal tissue obtained by skin biopsies from the site of the skin AF measurement, taken in patients on hemodialysis, DM and healthy controls, and its reproducibility is reasonably well showing a mean relative error in AF of 5.0% (74-76).

The autofluorescence reader

In 1996, a serendipitous finding of Jager et al. led to the development of the AF reader. Unexpectedly high baseline levels of skin AF were measured by a fluorescence microscope in diabetes patients compared to healthy controls, while measuring capillary sodium fluorescein leakage to investigate capillary permeability (77,78). This remarkable observation was the first step to the development of a non-invasive device reflecting tissue accumulation of AGEs. The so-called skin AF reader, eventually developed into the commercially available AGE reader (Diagnoptics Technologies, Groningen, The Netherlands) in 2006, figure 2a.

This technique is based on the fluorescence properties of certain AGEs and expresses the level of skin AF. The AGE reader illuminates a skin surface of ~ 4 cm^2 at the volar side of the arm, 10 cm below the elbow fold and uses an excitation UV-A light source with the intensity between 350 – 420 nm (maximum intensity at 370 nm). Emission light and reflected excitation light from the skin are measured with a spectrometer in the 300-600 nm range, figure 2b. Because of the influence of skin pigmentation, AF was computed by dividing the average light intensity of the emission spectrum by the average light intensity of the excitation spectrum. The level of skin AF is multiplied by 100 and is expressed in arbitrary units. In patients with dark skin, melanin in the epidermis will absorb a considerable part of the UV-A light source, resulting in lower reflection of the excitation UV light and less UV light penetration in the dermal layer of the skin, finally resulting in less fluorescence. Moreover, part of the fluorescent light itself will also be absorbed by melanin. Therefore patients with Fitzpatrick class V-VI skin type were excluded because of the limitation of the prototype AF reader to measure accurately in dark skin types and measurements with skin reflection below 10% were discarded as well. Recently, a newly developed AF reader has the capacity to measure in dark skin types (79). Another remark concerning skin AF measurement is the measurement of other fluorophores than fluorescent AGEs, acting as a confounder. Furthermore, there are non-fluorescent AGEs present in the skin as well, which may contribute to the overall effects of AGE accumulation.

Figure 2a. Skin autofluorescence reader in a clinical setting. Data from the integrated spectrometer of the AGE reader is passed on to a computer by USB connection and the measured spectrum and the skin autofluorescence value will be displayed on the screen.

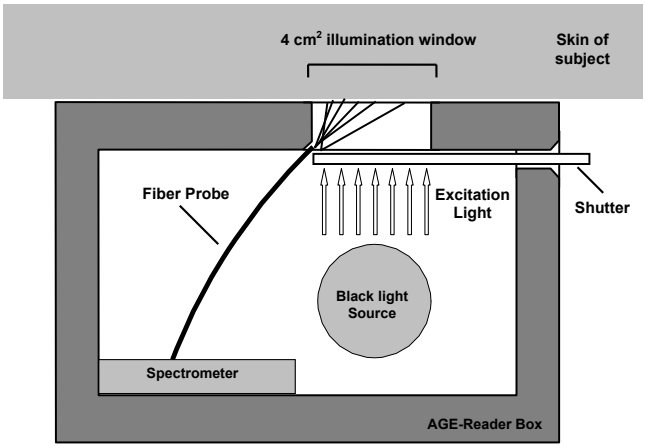

Figure 2b. Schematic diagram of the skin autofluorescence reader or AGE reader. The excitation light source is a black light TL tube. Two calibration measurements are performed with a closed shutter: one against a white reflection standard and one dark measurement. After opening of the shutter, the forearm skin of the subject is illuminated. Emission light and reflected excitation light from the skin within a range of 300 – 600 nm is transmitted by a fiber probe to the integrated spectrometer.

Objectives

An important aim of this thesis was to assess the possible predictive value of skin AF for (micro)vascular morbidity and mortality in two high risk patient groups: type 2 DM and ESRD patients. The studies assessed in *Part one* of the thesis included type 2 DM patients who were all participating in a shared-care project of the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC) Study.

We investigated the possible predictive value of skin AF for the development of microvascular complications in a type 2 DM patient group (Chapter 2). In an observational setting, we prospectively analyzed the relationship between the level of skin AF and the risk of developing microvascular complications of DM (retinopathy, neuropathy and nephropathy), both separately and combined.

Another objective of this thesis was to study whether glycemic control could predict the change in tissue AGE accumulation during time in type 2 DM (Chapter 3). Different integrated assessments such as the degree of glucose control, assessed by

sequential HbA1c measurements, were studied in relationship to skin AF in type 2 diabetes patients recruited from the earlier mentioned cohort participating in the ZODIAC study.

The ZODIAC-28 study was performed to investigate whether serum peroxiredoxin 4, as part of the antioxidant defense system, was associated with cardiovascular and all-cause mortality in the same population of type 2 diabetes patients as already mentioned (Chapter 4).

Finally, the ZODIAC-10 study was performed as a life expectancy study in type 2 DM with the aim to assess present-day life expectancy of these diabetes patients in the ongoing cohort of the ZODIAC study compared to the general Dutch population (Chapter 5).

Part two of this thesis, concerned another high risk patient group: patients with chronic kidney disease and/or ESRD who were on hemodialysis. Chapter 6 defines the relationship between AGEs, autofluorescence and renal function in an editorial comment with the inclusion of original data about the correlation between skin AF and the estimated glomerular filtration rate in type 2 DM patients.

Chapter 7 is a review article about skin AF as a measurement of AGEs and as a risk marker in chronic kidney disease. Finally, we studied the predictive role of skin AF on overall and cardiovascular mortality in a hemodialysis patient group (Chapter 8). These patients were recruited from the hemodialysis centre of the Isala Clinics in Zwolle, The Netherlands.

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PART I

CARDIOVASCULAR RISK AND ITS DETERMINANTS

IN TYPE 2 DIABETES MELLITUS

CHAPTER 2

SKIN AUTOFLUORESCENCE: A TOOL TO IDENTIFY TYPE 2 DIABETIC PATIENTS AT RISK FOR DEVELOPING MICROVASCULAR COMPLICATIONS

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Abstract

Objective Skin autofluorescence (AF) is a noninvasive measure of the level of tissue accumulation of advanced glycation endproducts, representing cumulative glycemic and oxidative stress. Recent studies have already shown a relationship between skin AF and diabetic complications, and its predictive value for total and cardiovascular mortality in type 2 diabetes mellitus. Our aim was to investigate the predictive value of skin AF for the development of microvascular complications in type 2 diabetes mellitus.

Research Design and Methods At baseline, skin AF of 973 well-controlled type 2 diabetes patients was noninvasively measured with an autofluorescence reader. The aggregate clinical outcome was defined as the development of any diabetes-associated microvascular complication of 881 surviving patients which was assessed at baseline and at the end of follow-up. Single endpoints were the development of diabetes associated retinopathy, neuropathy and (micro)albuminuria.

Results After a mean follow-up period of 3.1 years, baseline skin autofluorescence was significantly higher in patients who developed any microvascular complication, neuropathy or (micro)albuminuria, but not in those who developed retinopathy. Multivariate analyses showed skin AF as a predictor for development of any microvascular complication along with HbA1c, for development of neuropathy along with smoking, and for development of (micro)albuminuria together with sex, HbA1c and diabetes duration. Skin autofluorescence did not have predictive value for the development of retinopathy, albeit diabetes duration did.

Conclusions Our study is the first observation of skin autofluorescence measurement as an independent predictor for the development of microvascular complications in type 2 diabetes.

Introduction

Hyperglycemia, individual susceptibility and lifestyle are three key factors that play an important role in the development of microvascular disease in diabetes mellitus. One of the consequences of hyperglycemia and attendantly increased generation of free radicals is the increased formation of advanced glycation endproducts (AGEs), besides the increased polyol and hexosamine fluxes, and activation of protein kinase C, which all contribute to tissue damage in diabetes (1,2). Those AGEs can be described as the final products of slowly occurring non-enzymatic glycation of proteins that form cross-links with long-lived proteins such as collagen (the so called Maillard reaction). They may also accumulate as a result of oxidative stress-related glycoxidation and lipoxidation pathways.

In the Diabetes Control and Complications Trial (DCCT), long-term intensive treatment compared with conventional treatment of hyperglycemia in type 1 diabetic patients improved glycemic control, and delayed the progression of microvascular complications (3). The UK Prospective Diabetes Study and other prospective studies have also shown an association between hyperglycemia and increased risk of microvascular complications in type 2 diabetes (4-6). The DCCT Skin Collagen Ancillary Study Group showed the association of long-term intensive treatment of hyperglycemia, as compared with conventional treatment, with lower levels of AGEs in skin collagen and they showed that these AGE levels in skin biopsies predicted the risk of development or progression of microvascular disease in type 1 diabetes mellitus, even after adjustment for HbA1c (7,8).

A newly described noninvasive method to assess tissue AGEs concerns skin autofluorescence. This method is based on the specific fluorescence characteristics of AGEs and has been validated against specific AGE levels in skin biopsies in patients with diabetes or on hemodialysis, and in healthy control subjects (9,10).

Recently, the relationship between skin autofluorescence, reflecting AGE accumulation, and outcome has been studied in type 2 diabetes. Besides its relation with chronic diabetes complications (in cross-sectional analyses), skin autofluorescence has also shown independent predictive value for cardiovascular mortality and morbidity in patients with type 2 diabetes and in patients with end-stage renal disease undergoing hemodialysis (10-12)

In this study, we analyzed whether skin autofluorescence, as a marker of AGE accumulation, can predict the development of microvascular complications in a type 2 diabetic population.

Research Design and Methods

Patients

Between May 2001 and May 2002, 973 primary care type 2 diabetic patients were included in the study cohort and had a skin autofluorescence measurement. The included patients were all participating in a shared-care project of the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC) Study and have also been described elsewhere (11). During follow-up, data of 967 patients were analyzed for this study (6 patients were lost to follow-up). Eighty-six patients died before the end of follow-up and this subgroup will be addressed separately from the surviving 881 patients. Patients with a Fitzpatrick class V-VI skin type were excluded, because of the autofluorescence reader's limitation to measure accurately in dark skin types (13-15). All participating patients visited the outpatient clinic at least once a year. Follow-up ended at January 2005. All of the included patients had given their informed consent, and approval by the local ethics committee had been obtained.

Skin autofluorescence

The autofluorescence reader (prototype of the current AGE Reader; DiagnOptics, Groningen, the Netherlands) illuminates a skin surface of ~4 cm^2, guarded against surrounding light, with an excitation light source with peak intensity at ~370 nm. Emission light and reflected excitation light from the skin are measured with a spectrometer in the 300-600 nm range, using a glass fiber. AF was computed by dividing the average light intensity of the emission spectrum 420-600 nm by the average light intensity of the excitation spectrum 300-420 nm, multiplied by hundred and expressed in arbitrary units (AU). Skin autofluorescence of all patients was assessed at the volar side of the arm, 10 cm below the elbow fold. Six diabetes specialist nurses did the autofluorescence measurements with two identical

autofluorescence reader devices. The autofluorescence reader has been validated and more extensively been described in previous studies (9,11).

Data collection

Clinical data and laboratory results were obtained at the time of the baseline skin AF measurement. Serum creatinine, nonfasting lipids (total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides), and urinary albumin and creatinine were measured according to the standard laboratory procedures. HbA1c was measured with a Primus CLC-385 using boronate affinity chromatography and high-performance liquid chromatography (reference value 4.0 – 6.0%). Blood pressure measurement was a single measurement obtained after 5-minutes rest with the patient in seated position, using an aneroid device. At each visit to the outpatient clinic and at the end of follow-up, the absence or presence of retinopathy, neuropathy, and (micro) albuminuria was assessed.

Clinical end points

The aggregate clinical end point was the development of any diabetes-associated microvascular complication, which was defined as the presence of at least one of the following diabetes complications according to the American Diabetes Association definitions: retinopathy, neuropathy and/or (micro)albuminuria (16). The single clinical end points were described as the development of retinopathy, neuropathy or (micro)albuminuria. Retinopathy was determined by an ophtalmologist based on retinal photography. Presence of at least background retinopathy was assumed to imply retinopathy. Neuropathy was examined using a 5.07/10 g Semmes-Weinstein monofilament, applied on the dorsum of both feet at three different, non-callused areas (first toe, and first and fifth distal metatarsal bone). Neuropathy was considered in case of diminished sensibility, which was defined in case of at least two incorrect responses after 3 applications at each area (two real and one false application) (17,18). (Micro)albuminuria at baseline was defined as an albumin-to-creatinine ratio >2.5 mg/mmol for men and >3.5 mg/mmol for women in two subsequent urine samples or once in the year before baseline while using an ACE-inhibitor at baseline (19). Newly developed (micro)albuminuria at follow-up was defined as an albumin-

to-creatinine ratio >2.5 mg/mmol for men and >3.5 mg/mmol for women in two urine samples (one in the year before and one at the moment during follow-up) or an abnormal level of the albumin-to-creatinine ratio in the year before the end of follow-up whilst using an ACE-inhibitor at follow-up.

Statistical analysis

One-way ANOVA using posthoc multiple comparisons (with Bonferroni correction) was used to compare mean skin autofluorescence between subgroups of microvascular complications in the 881 surviving patients. Subgroups are as follows: 1) no microvascular complication at baseline or at follow-up, 2) no microvascular complication at baseline but a microvascular complication at follow-up and 3) a microvascular complication at baseline and at follow-up.

Univariate and multivariate multinominal regression analyses were performed to determine the relationship of skin autofluorescence to the presence or development of microvascular disease. Patients without signs of microvascular complications at baseline or at follow-up formed the reference categories in these calculations. In the multivariate analyses, we controlled for potential confounding risk factors for the development of microvascular complications which were derived from the UKPDS findings, including sex, diabetes duration, HbA1c, current smoking, systolic blood pressure, HDL cholesterol, LDL cholesterol and triglycerides with the addition of BMI (4).

Odds ratios (ORs) (CI 95%) for skin autofluorescence were calculated in the univariate and multivariate analyses. *P* values <0.05 were considered statistically significant.

Results

The baseline characteristics of the surviving study population including mean skin autofluorescence of the total group are shown in Table 1. Mean age of our study population was 66 years, 46% of whom were male, with a relatively short median diabetes duration of 4.0 years (interquartile range 1.5 – 8.1 years). Eighty-five percent of this study population with well-controlled diabetes was on a diet and/

or oral agents; the other 15% of patients received insulin or combined insulin/oral agent treatment.

Table 1. Characteristics of the type 2 diabetic patients.

Characteristic *n*	881
Age (years)	66 ± 11
Sex (male/female)	406/475
Smoking (%)	19
BMI (kg/m²)	29.4 ± 4.8
Systolic blood pressure (mmHg)	146 ± 20
Diabetes duration (years)	4.0 (1.5-8.1)
HbA1c (%)	6.6 (6.0-7.6)
Creatinine (µmol/l)	95 ± 19
Creatinine clearance (ml/min)	77 ± 27
Urinary albumin-to-creatinine ratio	1.41 (0.76-3.79)
Total cholesterol (mmol/l)	5.2 ± 1.0
HDL cholesterol (mmol/l)	1.3 ± 0.3
LDL cholesterol (mmol/l)	2.9 ± 0.9
Triglycerides (mmol/l)	2.1 (1.4-2.9)
Microvascular disease (%)	50
Retinopathy (%)	19
Neuropathy (%)	24
(Micro)albuminuria (%)	24
Macrovascular disease (%)	37
Skin autofluorescence (total group) (AU)	2.74 ± 0.7

Values are mean ± SD or median (interquartile range) unless otherwise indicated. Reference values of the laboratory: HbA1c 4.0-6.0 %, creatinine 70-110 µmol/l, creatinine clearance (Cockcroft-Gault formula) 80-120 ml/min, urinary albumin-to-creatinine ratio 0-2.5, total cholesterol 3.5-5.0 mmol/l, HDL cholesterol 0.9-1.7 mmol/l, LDL cholesterol 3.6-4.4 mmol/l, and triglycerides 0.6-2.2 mmol/l.

In the 881 survivors, the prevalence of retinopathy, neuropathy and (micro) albuminuria at baseline was 19, 24 and 24%, respectively, resulting in an overall percentage of patients with a diabetes associated microvascular complication of 50%.

Table 2 shows the mean baseline skin autofluorescence of the 881 survivors subdivided in groups with continued absence or presence or the development of microvascular complications at follow-up. During a median follow-up period of 3.1

years, 61 patients (7.0%) developed retinopathy; their baseline skin autofluorescence did not differ from skin autofluorescence levels of patients who did not show or already had retinopathy at baseline. However, skin autofluorescence was higher in the patient groups who developed neuropathy or (micro)albuminuria compared to those without these complications. At follow-up, newly developed neuropathy was diagnosed in 7.5% and newly developed (micro)albuminuria was found in 10.1% of patients; 12.5% of the population developed at least one microvascular complication. Skin autofluorescence at baseline was also significantly higher in the patient groups that developed any microvascular complication or who already had a microvascular complication at baseline compared with patients who did not develop any microvascular disease.

Table 2. Mean ± SD skin autofluorescence at baseline and mean differences between groups.

	A	B	C	B vs A	C vs A	C vs B
Microvascular complication	**t_0: absent t_{fu}: absent**	**t_0: absent t_{fu}: present**	**t_0: present t_{fu}: present**			
Retinopathy	2.69 ± 0.73	2.88 ± 0.74	2.91 ± 0.72	0.20	0.22	0.02
n	647	61	169	(-0.04 to 0.43)	(0.07 to 0.37)	(-0.24 to 0.29)
P				0.14	0.002	1.00
Neuropathy	2.67 ± 0.72	2.93 ± 0.75	2.88 ± 0.75	0.26	0.21	-0.05
n	596	66	215	(0.03 to 0.49)	(0.07 to 0.35)	(-0.29 to 0.20)
P				0.019	0.001	1.00
(Micro) albuminuria	2.62 ± 0.68	2.91 ± 0.67	2.97 ± 0.83	0.28	0.34	0.06
n	570	87	207	(0.09 to 0.48)	(0.20 to 0.48)	(-0.16 to 0.28)
P				0.002	<0.001	1.00
Any	2.52 ± 0.69	2.86 ± 0.66	2.88 ± 0.75	0.34	0.36	0.01
n	322	109	441	(0.15 to 0.53)	(0.23 to 0.48)	(-0.17 to 0.20)
P				<0.001	<0.001	1.00

Data are means ± SD of skin autofluorescence in AUs within the group or mean differences between groups (95% CI) (ANOVA with Bonferroni correction). t_0, baseline; t_{fu}, follow-up.

Multinominal logistic regression analysis showed that skin autofluorescence was a strong predictor of the development of the aggregate of microvascular complications [OR 2.05 (95% CI 1.51-2.80), p<0.001]. Skin autofluorescence was significantly associated with the development of retinopathy [1.42 (1.01-1.99), p=0.042], neuropathy [1.59 (1.15-2.19), p=0.005], and (micro)albuminuria [1.73 (1.28-2.34), p<0.001]. After correction for the confounding risk factors, baseline skin autofluorescence still appeared to be significantly associated with the development of these end points, except for retinopathy [1.21 (0.83-1.74), p=0.32] (Table 3). Diabetes duration at baseline was the only significant independent variable for the development of retinopathy in this multivariate analysis [1.10 (1.06-1.15), p<0.001]. Surviving smokers less often developed neuropathy compared with non-smokers. In the nonsurviving group (86 patients), 70% had a microvascular complication at baseline; there were 23 non-surviving smokers. Seventy percent of the non-surviving smokers already had a microvascular complication at baseline, and 13% of the non-surviving smokers developed a microvascular complication before they died.

When baseline skin autofluorescence levels are categorized in subgroups of practically feasible levels of skin autofluorescence (three categories in rounded tertiles: skin autofluorescence < 2.35 AU, 2.35 ≤ skin autofluorescence < 3.00 AU, skin autofluorescence ≥ 3.00 AU); those in the category skin autofluorescence ≥3.00 AU do have a higher chance to develop a microvascular complication compared to patients with a lower skin autofluorescence level (Table 4).

Table 3. Variables related to the development of microvascular complications in type 2 diabetes by multinominal logistic regression analysis.

Variables	Any microvascular complication		Retinopathy		Neuropathy		(Micro)albuminuria	
	P	OR 95% CI	*P*	OR 95% CI	*P*	OR 95% CI	*P*	OR 95% CI
Skin AF	<0.001	2.02 1.45-2.81	0.32	1.21 0.83-1.74	0.026	1.50 1.05-2.14	<0.001	1.88 1.36-2.61
Sex	0.02	0.55 0.33-0.90	0.91	0.97 0.53-1.75	0.78	1.09 0.61-1.93	0.001	0.42 0.25-0.71
HbA1c	0.004	1.30 1.09-1.55	0.13	1.18 0.95-1.45	0.87	1.02 0.82-1.26	0.034	1.21 1.01-1.44
Diabetes duration	0.66	1.01 0.96-1.06	<0.001	1.10 1.06-1.15	0.032	1.04 1.00-1.08	0.04	0.95 0.90-0.997
Smoking	0.07	0.56 0.29-1.05	0.09	0.48 0.21-1.11	0.011	0.29 0.11-0.75	0.96	1.02 0.56-1.85
Systolic bloodpressure	0.43	1.01 0.99-1.02	0.39	1.01 0.99-1.02	0.49	1.01 0.99-1.02	0.18	1.01 0.996-1.02
LDL cholesterol	0.48	1.09 0.85-1.40	0.66	0.93 0.69-1.27	0.35	0.87 0.64-1.17	0.30	1.15 0.89-1.49
HDL cholesterol	0.26	0.62 0.27-1.43	0.36	0.63 0.23-1.70	0.081	0.41 0.15-1.12	0.40	0.38 0.15-0.96
Triglycerides	0.54	0.94 0.78-1.14	0.41	0.91 0.72-1.15	0.85	0.98 0.79-1.22	0.19	0.87 0.71-1.07
BMI	0.27	1.03 0.98-1.08	0.33	1.03 0.97-1.09	0.56	0.98 0.93-1.04	0.39	1.02 0.97-1.08

AF, autofluorescence measured with the autofluorescence reader (see Research design and methods).

Table 4. Prediction of newly developed microvascular complications subdivided into three skin autofluorescence (AF) groups.

Microvascular complication	*n**	Skin AF < 2.35 AU	2.35 ≤ Skin AF < 3.00 AU	Skin AF ≥ 3.00 AU
Retinopathy	708	15/241 (6.2)	18/251 (7.2)	28/216 (13.0)
Neuropathy	662	11/219 (5.0)	27/247 (10.9)	28/196 (14.3)
(Micro)albuminuria	657	18/225 (8.0)	31/253 (12.3)	38/179 (21.2)
Any	431	23/161 (14.3)	41/167 (24.6)	45/103 (43.7)

Data are n (%) of newly developed microvascular complications of subgroups compared to the group who did not develop a microvascular complication. *Patients who did not have a complication at baseline. Subgroups of skin AF are tertiles rounded to a practical level.

Conclusions

Our study provides the first evidence that skin autofluorescence is an independent predictor of the development of microvascular complications in a population of patients with well-controlled type 2 diabetes. Separately, this also holds for the development of neuropathy and (micro)albuminuria (and in univariate analysis for retinopathy). This noninvasive marker of tissue AGE accumulation may reflect the deleterious effects of long-term glycemic and oxidative stress. Meerwaldt et al. recently showed that skin autofluorescence is a predictor of 5-year coronary heart disease and mortality in diabetes (12). The present study shows that skin autofluorescence also has a predictive value for the development of microvascular complications that, in the analysis of this study, is superior to that of many other commonly used risk predictors, such as diabetes duration and HbA1c, in type 2 diabetes. This conclusion is applicable for primary care type 2 diabetic patients treated according to current standards, which is the large majority of type 2 diabetes patients in the Netherlands.

The DCCT/EDIC (Epidemiology of Diabetes Interventions and Complications) substudy already showed the predictive value for skin AGE levels obtained from skin biopsies for the progression of microvascular complications in patients with type 1 diabetes (8). Our study population consisted of type 2 diabetic patients with skin AGE level assessment by means of a noninvasive, rapid method. Another difference is that the DCCT/EDIC substudy investigated the development as well as the progression

of microvascular complications. The limited follow-up period; the low rate of clearly classifiable progression of the microvascular complications, especially retinopathy; and the confounding role of introduced medication made us decide to restrict our study to the evaluation of the development of microvascular complications and not to address progression of these diabetes complications.

In retinopathy, skin autofluorescence turned out to have no prognostic value in the multivariate analysis. Possible explanations are the short follow-up period and the smaller amount of patients who developed retinopathy versus the other complications. Moreover, the different pathophysiologic mechanisms of microvascular damage in the different organs (retina, kidneys and neurons) could play a role in the differences in incidence rates of outcomes. In particular, the pathobiology of retinopathy might be different from that of the kidney and neurologic system as a result of a different role of vascular endothelial growth factor as a possible mediator for proliferation (20).

(Micro)albuminuria is an early clinical sign of diabetic nephropathy; when left untreated, it predicts a high risk for the development of progressive renal damage, which eventually may lead to end stage renal disease. Progressive renal disease is also associated with a vastly increased cardiovascular risk. This study defined (micro) albuminuria as a sign of microvascular complication with the intention to reflect early stages of diabetic nephropathy.

In the predictive analyses, the non-surviving patients were excluded from the analyses. These non-survivors had markedly increased skin autofluorescence values, but they also had a very high prevalence of microvascular complications at baseline (70%), so this does not reduce the strength of the relation between skin autofluorescence and microvascular complications.

Ethnicity is one of the mentioned UKPDS confounding risk factors for the development of microvascular disease. Because of the limitation of measuring skin autofluorescence in dark skin types associated with the prototype of the AGE reader used in the present study, individuals with dark skin had to be excluded. Over 95% of the participants were Caucasian; therefore, ethnicity was not taken into account in the analyses. Further developments of the AGE reader may hopefully enable measurements in dark skin type in future investigations.

Lutgers et al. previously described the other limitations of the autofluorescence reader as a marker of tissue AGE accumulation: non-fluorescent AGEs will not be measured with the autofluorescence reader, and other tissue components that fluoresce in the same range of wavelength might be confounders (11).

In conclusion, our study confirms skin autofluorescence as a helpful clinical method to identify type 2 diabetic patients at risk for (developing) any microvascular complication, neuropathy and (micro)albuminuria. Further investigation with longer follow-up needs to be done to assess whether skin autofluorescence is a factor in the development of diabetic retinopathy and to assess the relationship of skin autofluorescence and the progression of microvascular complications. Its non-invasive and time-saving application makes the autofluorescence reader an easy clinical tool that is useful in the outpatient clinic in risk assessment and for monitoring changes in accumulation of tissue AGEs reflecting long-term glycemic stress.

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Conflicts of interest

R. Graaff and A.J. Smit are founders of DiagnOptics B.V., The Netherlands, manufacturer of the AGE-Reader, which is based on the prototype used in the present article.

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CHAPTER 3

SKIN ADVANCED GLYCATION ENDPRODUCTS ACCUMULATION IS POORLY REFLECTED BY GLYCEMIC CONTROL IN TYPE 2 DIABETIC PATIENTS (ZODIAC-9)

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Abstract

Background Glycemic memory can be reflected by tissue accumulation of advanced glycation endproducts (AGEs). In type 1 diabetes mellitus (T1DM) patients, hemoglobine A1c (HbA1c) levels over various time periods poorly predicted the accumulation of different AGEs in skin biopsies. Our aim was to investigate whether HbA1c assessments can predict the change in skin AGEs during time in type 2 diabetes mellitus (T2DM).

Methods We included 452 T2DM patients participating in a shared-care setting, who are screened annually for HbA1c and diabetic complications. Baseline and follow-up levels of skin AGEs were assessed with a validated noninvasive autofluorescence (AF) method which is based on the fluorescence characteristics of certain AGEs.

Results Our study population had a mean age of 65 years and 54% were female. After a mean follow-up duration of 3.3 years, linear regression analyses showed weak relationships among different assessments of HbA1c (baseline, maximum, mean and variance of HbA1c) and skin AF at follow-up. Baseline skin AF and age were predictors of skin AF at follow-up, but diabetes duration, smoking, and creatinine were of less or no predictive value for skin AF at follow-up.

Conclusions In our T2DM population, integrated HbA1c assessments over years poorly predict the change in skin AGE level measured by skin AF. These findings agree with results in patients with T1DM. This suggests either the need for longer exposure to glucose disturbances to change tissue AGEs or other mechanisms, such as oxidative stress, leading to AGE accumulation.

Introduction

Short-term glycemic memory can be reflected by hemoglobine A_{1c} (HbA1c), which represents the degree of glycemic control over the last 6 to 8 weeks. Another more long-term glycemic index encompasses the level of tissue accumulation of advanced glycation end products (AGEs) (1). These stable end products of glycation of proteins are formed nonenzymatically in the Maillard reaction from Amadori products such as HbA1c. AGEs can also be formed by reactive carbonyl compounds (e.g. glyoxal, methylglyoxal, arabinose, glycoaldehyde) in conditions of enhanced oxidative stress in general. AGEs form cross-links in and accumulate on long-lived proteins such as skin collagen, which has an estimated lifetime of 15 – 20 years. Quantization of these collagen-bound AGEs could provide information about cumulative glycemic and oxidative stress over several years. Part of this metabolic process is a consequence of poor metabolic control over a considerable period, probably years.

Important evidence regarding the relationship between poor metabolic control and the development or progression of diabetic complications has been found earlier in T1DM in the Diabetes Control and Complications Trial (DCCT) and in T2DM in the United Kingdom Prospective Diabetes Study (UKPDS) study during the nineties (2,3). Relevance of the glycated collagen products compared to HbA1c, both as markers of diabetic complications, has been investigated in a DCCT – Epidemiology of Diabetes Interventions and Complications (EDIC) substudy (1). Long-term intensive treatment compared to conventional treatment of type 1 diabetes patients resulted in lower skin collagen glycation, glycoxidation, and cross-links. The accumulation of collagen AGEs, as measured in skin biopsies, explained an unexpectedly high percentage of variance in the incidence of diabetic complications, also after adjustment for HbA1c levels. Monnier et al. also found that the AGE accumulation in skin biopsies of type 1 diabetic patients was poorly predicted by HbA1c levels over several different time periods (2).

Advanced glycation end product accumulation can be quantified by tissue measurements, invasively by skin biopsies, but also noninvasively with an

autofluorescence reader (AFR) or currently AGE reader. This newly developed device measures certain tissue AGEs, with the concept of reflecting metabolic control over several years, and has already been established as a risk marker for micro- and macrovascular complications and mortality in T2DM (4,5). Controlling glycemic and metabolic status is an important issue in preventing long-term diabetic complications and it is not clearly defined which marker for metabolic control over a certain period is the best predictor in relation to the development of chronic diabetic complications. We hypothezised that the accumulation of AGEs in type 2 diabetes patients can be predicted by the course of HbA1c during a certain period prior to the skin autofluorescence (AF) measurement. The aim of our study was to investigate to what extent skin AF, reflecting tissue AGE accumulation, can be predicted by different integrated assessments of HbA1c in type 2 diabetic patients.

Methods

Study Population

Subjects were recruited from a large type 2 diabetes cohort participating in the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC)-study (6). Baseline skin AF measurements were performed in a cohort of 973 patients between May 2001 and May 2002 (7). At the end of follow-up, from June 2004 untill October 2005, a second skin AF was assessed randomly in 452 patients from the initial population of 973 patients who were still visiting the diabetes outpatient clinic. Because of the limitation of the applied AFR to measure accurately in dark skin types, patients with a Fitzpatrick class V-VI skin type were excluded from the beginning. All participating patients visited the outpatient clinic annually and all of them had given informed consent. Approval by the local ethical committee had been obtained.

Autofluorescence Reader

Assessment of tissue AGEs by the AFR device, a prototype of the current AGE reader (DiagnOptics Technologies BV, Groningen, The Netherlands), is based on a technique that enlists the fluorescence properties of certain AGEs, as mentioned before,

and expresses the level of skin AF. This non-invasive measurement is obtained by positioning the ventral side of the lower arm on a window in a box containing an excitation ultraviolet A light source with a peak wavelength around a 370 nm excitation light, integrated over the 300 to 420 nm range. Reflected light from the skin and emission light in the 420 to 600 nm range are measured with an integrated spectrometer. Dividing the average light intensity of the emission spectrum by the average light intensity of the excitation spectrum expresses the skin AF level in arbitrary units (AU). To calculate skin reflection, white reference measurements (with a white Teflon block, assuming 100% reflectance) were performed before every measurement. Over all AF measurements, the mean age-corrected AF per measuring month, per examiner, and per AFR system did not differ significantly. The AFR has been described more in detail elsewhere (8).

Clinical data and Laboratory Assessments

Clinical data were obtained on the date of the first skin AF measurement; these data were derived from the Diabetic Electronic Monitoring System (DEMS) containing data of all patients in the shared-care project (6). Laboratory assessments were measured according to the standard hospital procedures of the Isala Clinics, Zwolle, The Netherlands. HbA1c was measured with a Primus CLC-385 using boronate affinity chromatography and high-performance liquid chromatography (reference value 4.0 – 6.0%). The presence of microvascular disease was defined as the occurrence of at least one of the following diabetic complications: retinopathy, neuropathy and/or nephropathy. We used the albumin-to-creatinine ratio (ACR) in our definition of nephropathy. The relationship between skin AF and ACR was shown in the original study population, and the predictive value of skin AF for the development of nephropathy has been studied as well (4,7). Macrovascular disease was defined as the presence of at least one aspect of the following cardiovascular complications: coronary heart, cerebrovascular and/or peripheral vascular disease. All complications are described in detail elsewhere (7).

Skin AF assessments were performed at baseline and at the end of follow-up.

Statistical analysis

Comparing means of skin AF between patients with and without a diabetic complication was performed by analysis of variance.

Linear regression was used to determine the relationship between skin AF at follow-up and various HbA1c measures. The different integrated assessments of HbA1c used in the analyses were: the variance of HbA1c, mean HbA1c, maximum HbA1c, and HbA1c at baseline. Mean and variance of HbA1c were calculated over the annually assessed HbA1c measurements between baseline and the end of follow-up. Maximum HbA1c is the highest HbA1c level of the annually assessed HbA1c measurements between baseline and the end of follow-up.

Adjustment for baseline skin AF was performed in all the analyses. In multivariate regression, in addition to baseline skin AF, adjustments for age, diabetes duration, creatinine, and smoking were made (all variables were assessed at baseline). These variables are all independently related to skin AF and could all affect the rate of formation and accumulation of AGEs during years (7).

Results

Baseline characteristics are shown in Table 1. Mean age was 65 years with 255 (54%) female patients. The mean follow-up duration was 3.3 ± 0.4 years. At baseline, patients were well-controlled with a mean HbA1c of 6.8 ± 1.2 % and mean HbA1c at follow-up was 7.0 ± 1.0 %. Patients with either a microvascular or a macrovascular complication had higher levels of skin AF at baseline (Table 2).

The total amount of annually collected samples of HbA1c per patient was minimal 3 and maximal 5; the mean of collected samples of HbA1c was 4.2.

Table 1. Baseline characteristics.

Patient characteristic	N = 452
Age (years)	65 (11)
Gender (F/M)	244/208 (54%/46%)
Smoking (%)	19
Body Mass Index (kg/m^2)	29.5 (4.9)
Systolic bloodpressure (mmHg)	145 (20)
Diastolic bloodpressure (mmHg)	81 (10)
Diabetes duration (years)	3.6 (1.5 – 7.5)^a
HbA1c (%)	6.8 (1.2)
Creatinine (µmol/l)	94 (17)
Total cholesterol (mg/dl)	197 (37)
Microvascular disease (%)	46
Macrovascular disease (%)	35
Skin AF at t0 (AU)	2.71 (0.69)
Skin AF at t follow up (AU)	2.79 (0.77)

Values are expressed as mean (SD). ^{a}Median and interquartile range. Reference values of the laboratory: HbA1c 4.0-6.0 %, creatinine 70-110 µmol/l, total cholesterol 135-193 mg/dl. AU, arbitrary units.

Table 2. Mean skin AF ± standard deviation (arbitrary units) of patients with or without a micro/macrovascular complication.

Complication	**Yes *(n)***	**No *(n)***	***p* value**
Microvascular	2.81 ± 0.70 (208)	2.63 ± 0.67 (244)	0.005
Macrovascular	2.91 ± 0.74 (156)	2.61 ± 0.63 (296)	<0.001

The relationship between skin AF at follow-up (adjusted for the also presented skin AF at baseline) and the various assessments of HbA1c are shown in Table 3. Skin AF at follow-up showed weak, but significant relationships with all different integrated assessments of HbA1c: regression coefficients < 0.1; $p \leq 0.025$ [with addition of baseline skin AF: overall adjusted $R^2 \sim 0.45$; $p<0.001$]. Table 4 shows the linear regression analyses of skin AF at follow-up versus different integrated assessments of HbA1c with adjustment for baseline skin AF, age, diabetes duration, creatinine and smoking. In all analyses adjusted R^2 of skin AF at follow-up was 0.48 ($p<0.001$), although the regression coefficients of all the different integrated HbA1c assessments were low (maximum 0.069) and, therefore, added little prognostic value to skin AF level at follow-up. Skin AF level at baseline proved to be the best predictor of skin AF at follow-up (regression coefficient = 0.65 for mean HbA1c, maximum HbA1c, HbA1c at baseline, and 0.66 for the variance of HbA1c; $p<0.001$). Age and creatinine were of less predictive value than skin AF at baseline. In these models diabetes duration and smoking were not of any prognostic value for skin AF at follow-up.

Table 3. Linear regression models of skin AF at follow up (adjusted for skinAF at t0) versus different integrated assessments of HbA1c.

HbA1c assessment Baseline skin AF	Regression-coefficient	95% Confidence interval	*p*-value
Variance of HbA1c	0.067	0.020 – 0.114	0.005
Baseline skin AF	0.745	0.669 – 0.822	<0.001
Mean HbA1c	0.075	0.018 – 0.133	0.010
Baseline skin AF	0.728	0.650 – 0.806	<0.001
HbA1c max	0.053	0.014 – 0.091	0.007
Baseline skin AF	0.735	0.658 – 0.812	<0.001
HbA1c at baseline	0.052	0.007 – 0.098	0.025
Baseline skin AF	0.734	0.656 – 0.811	<0.001

Table 4. Multivariate Linear regression models of skin AF at follow up (adjusted for baseline skinAF, age, diabetes duration, creatinine and smoking) versus different integrated assessments of HbA1c.

HbA1c assessment - co-variates	Regression coefficient	95% Confidence interval	*p-value*
Variance of HbA1c	0.069	0.024 – 0.115	0.003
- **skin AF t0**	0.658	0.577 – 0.739	<0.001
- **age**	0.012	0.007 – 0.018	<0.001
- **diabetes duration**	0.003	-0.006 – 0.012	0.512
- **creatinine**	0.004	0.000 – 0.007	0.027
- **smoking**	0.064	-0.070 – 0.198	0.350
Mean HbA1c	0.069	0.011 – 0.127	0.021
- **skin AF t0**	0.647	0.565 – 0.729	<0.001
- **age**	0.012	0.007 – 0.018	<0.001
- **diabetes duration**	0.000	-0.009 – 0.010	0.959
- **creatinine**	0.003	0.000 – 0.006	0.040
- **smoking**	0.067	-0.067 – 0.201	0.327
HbA1c max	0.052	0.014 – 0.090	0.007
- **skin AF t0**	0.650	0.569 – 0.732	<0.001
- **age**	0.013	0.007 – 0.018	<0.001
- **diabetes duration**	0.001	-0.008 – 0.010	0.851
- **creatinine**	0.003	0.000 – 0.006	0.037
- **smoking**	0.064	-0.070 – 0.198	0.346
HbA1c at baseline	0.052	0.007 – 0.097	0.022
- **skin AF t0**	0.649	0.567 – 0.731	<0.001
- **age**	0.012	0.007 – 0.018	<0.001
- **diabetes duration**	0.001	-0.008 – 0.010	0.848
- **creatinine**	0.004	0.001 – 0.007	0.023
- **smoking**	0.066	-0.069 – 0.200	0.339

Discussion

This study showed that skin AF, reflecting tissue AGE accumulation and proposed as an exponent of glycemic long-term memory, is poorly predicted by the degree of short-term glycemic control in a type 2 diabetes population. The variance, mean, maximum and baseline measurements of HbA1c during a 3.3-year period prior to the skin AF measurement at the end of the follow-up period, were all of little value for predicting the change in AGE accumulation. These findings confirm the observations by Monnier and colleagues in the DCCT-EDIC cohort in T1DM (2). In the DCCT – EDIC substudy analyses of skin collagen variables against HbA1c levels at various time points regarding the date of biopsy were adjusted for age and diabetes duration only (1). In our cohort, age and skin AF at baseline were the most pronounced factors that influenced skin AF at follow-up, which is not surprising. A stable or small increase of the accumulation of AGEs was seen during the relatively short follow-up period. This also confirms the slow process of formation and accumulation of AGEs in human tissue, but could also be due to the on average well-controlled study population (mean HbA1c of 6.8%). The mean increase in skin AF of 0.08 AU over 3.3 years is also in line with the similarly small age decade-related differences in skin AF levels in our previous cross-sectional study in the same cohort (7).

Renal function as reflected by serum creatinine, affects the capacity for AGE removal and, therefore, plays an independent role in the accumulation of tissue AGEs. In the present study, it was indeed confirmed that serum creatinine contributes to the change in skin AF at follow-up. Cross-sectional data showed that smoking resulted in increased skin AF levels in both T2DM and controls, but in the present longitudinal analyses, smoking at baseline did not correlate to skin AF levels at follow-up (7). This could be due to the short follow-up period and/or to the strict diabetes treatment regimen of patients participating in the shared care project, which included stimulation of patients to stop smoking. The percentage of smokers was low in our cohort. Because tobacco smoke is a source of precursors of AGEs and free radicals, which both enhance AGE formation and accumulation, cessation of smoking could result in stabilization or probably decrease the rate of AGE accumulation (9,10).

In our study, unfortunately, no data were available on the effects of short-term glycemic variability nor on the degree of oxidative stress. Hyperglycemia is a well-known endogenous source of oxidative stress (11,12). Monnier and associates confirmed that glucose fluctuations during postprandial periods exhibited a more specific triggering effect on oxidative stress than chronic sustained hyperglycemia in type 2 diabetic patients (13). However, no relationship was seen between high glucose variability and elevated levels of oxidative stress in patients with type 1 diabetes, although these patients had higher urinary levels of oxidative stress than healthy controls (14). Markers of oxidative stress will be measured in future studies to determine the degree of oxidative stress and to study its relationship with skin AF, reflecting the accumulation of AGEs, and glycemic variability.

The relatively small numbers of total collected samples of HbA1c per patient (due to the short follow-up period) and the overall good glycemic control in our study population are two other limitations of our study; the latter could be considered as a limitation in assessing the rate of AGE accumulation. We cannot exclude that extending the range of glycemic control could have resulted in a stronger contribution of the HbA1c parameters to skin AF. Perhaps a stronger relationship would also have been found in poorly controlled subjects studied over a longer time period.

This study confirmed that the level of skin AF is partly determined by glycemic control defined as different assessments of HbA1c. Information about skin AGE levels could be of more important value than HbA1c levels for the development of diabetic complications, which could make the noninvasive AFR to a practical adjuvant in clinical practice. Follow-up studies have already shown the usefulness of skin AF as a new marker in predicting diabetic complications which turned out to be a stronger predictor than HbA1c. This was found for microvascular complications as well as for macrovascular morbidity and mortality (4,5). The mainstay of diabetes management is the prevention of chronic complications, especially cardiovascular disease. The presence of a considerable number of patients with micro- and macrovascular disease in our well-controlled study population at baseline supports the consensus that HbA1c parameters, reflecting at best medium-term glycemic control, cannot be

considered as a substitute for the use of skin AF, reflecting the accumulation of AGEs with its vascular damaging effects.

Conclusions

In conclusion, the present type 2 diabetes study showed glycemic control, measured by different assessments of HbA1c, as a small contributor to AGE accumulation expressed as skin AF. This poor relationship between HbA1c and skin AF in this group of type 2 diabetes mellitus patients is in agreement with the results of the DCCT in T1DM patients. Skin AGE levels could reflect the gravity of vascular damage even better than glycemic control by HbA1c. The finding also suggests other mechanisms for increased AGE accumulation, such as oxidative stress.

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Conflicts of interest
A.J. Smit is one of the founders of DiagnOptics B.V., The Netherlands, manufacturer of the AGE-Reader, which is based on the prototype used in this article.

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CHAPTER 4

SERUM PEROXIREDOXIN 4 AND MORTALITY IN PATIENTS WITH TYPE 2 DIABETES (ZODIAC-28)

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Submitted

Abstract

Background Oxidative stress plays an underlying pathophysiologic role in the development of diabetes complications. The aim of this study was to investigate peroxiredoxin 4 (Prx4), a proposed novel biomarker of oxidative stress, and its association with and capability as a biomarker in predicting mortality in patients with type 2 diabetes mellitus (T2DM).

Methods and results Prx4 was assessed in baseline serum samples of 1161 well-controlled T2DM patients (mean age 67). After a median follow-up period of 5.8 years, 327 (28%) patients died; 137 cardiovascular deaths (42%). Increased levels of Prx4 were associated with higher rates of cardiovascular and all-cause mortality. The Cox proportional hazard models added the following variables: Prx4 (model 1); age and gender (model 2), and BMI, serum creatinine, smoking, diabetes duration, systolic blood pressure, cholesterol-HDL ratio, history of macrovascular complications, and albuminuria (model 3) as additional potential confounders. Hazard ratios (95% confidence interval) for cardiovascular mortality were 1.93 (1.57 – 2.38), 1.75 (1.39 – 2.20), and 1.63 (1.28 – 2.09) for models 1, 2 and 3, respectively. Hazard ratios for all-cause mortality were 1.73 (1.50 – 1.99), 1.50 (1.29 – 1.75), and 1.44 (1.23 – 1.67) for models 1, 2 and 3, respectively. Addition of Prx4 to a model containing the traditional risk factors results in improved prediction of cardiovascular and all-cause mortality.

Conclusions Higher levels of serum Prx4 are independently associated with an increased risk of cardiovascular and all-cause mortality in T2DM and it may have the potential to become a promising cardiovascular biomarker in this patient group.

Introduction

Hyperglycemia and lifestyle factors are key factors in the development of diabetes-related morbidity and mortality (1-5). The hyperglycemic state is associated with higher levels of oxidative stress through the formation of excessive reactive oxygen species, which in turn activate the inflammatory cascade (6,7). This process is thought to play an important underlying pathophysiologic role in the development of diabetes complications, both microvascular and cardiovascular (8).

All aerobic organisms have a number of antioxidant proteins as a protection mechanism against oxidative stress. The antioxidant defense system comprises enzymatic and non-enzymatic systems, that can scavenge oxygen radicals, repair and remove damaged intracellular components. Peroxiredoxin enzymes are thiol-dependent peroxidases and part of a family of proteins present in aerobic organisms, responsible for the degradation of endogenously generated peroxides (9-11). These peroxiredoxin family members are distributed in the cytosol, mitochondria, peroxisomes and in plasma, which are all potential sites of free oxygen radical production (9). Overexpression or upregulation of peroxiredoxins has been found to be associated with higher levels of oxidative stress, which suggests a secondary respons of peroxiredoxins to oxidative stress (9,12-14). Six isoforms of peroxiredoxins have been described in mammals and peroxiredoxin 4 (Prx4) is the only isoform detectable in serum, because it is secreted by the endoplasmic reticulum of endothelial cells (15). Animal models of diabetes mellitus have shown changes of expression or oxidation state of Prx4 in pancreatic islet cells (14,16-18). No serum levels of Prx4 have been measured before in animal models neither in human with type 2 diabetes mellitus (T2DM). Serum levels of Prx4 have been proposed as a biomarker of oxidative stress in patients with rheumatoid arthritis and sepsis (19-21). The aim of this study was to prospectively investigate whether Prx4 is independently associated with cardiovascular and all-cause mortality and whether it could potentially be a new cardiovascular biomarker in patients with T2DM.

Materials and Methods

Study group and design

The included patients in our study are type 2 diabetic patients participating in a shared care project of the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC) Study. This project started in 1998 in Zwolle, The Netherlands and is still ongoing. In short, the objective of the ZODIAC Study was to investigate the effects of a shared-care project for type 2 diabetic patients. Sixty-one general practitioners participated. The ZODIAC Study was approved by the local medical ethics committee and all patients gave their informed consent (22). The present study incorporates two cohorts from the ZODIAC Study: one cohort started at the beginning in 1998 and the other in 2001. The latter was formed in order to investigate the predictive capability of skin autofluorescence as a marker of accumulation of advanced glycation end products for diabetes-related complications and mortality. This study has been described in detail before (23). The first cohort contained 1143 patients and the second cohort included 973 patients. There were 427 patients present in both cohorts, leaving a combined cohort of 1689 unique patients.

Data collection

Clinical data were obtained at the time of inclusion in the ZODIAC Study, which consisted of a complete medical history including macrovascular complications, medication use, diabetes duration and smoking history. Patients were considered to have macrovascular complications when they had a history of angina pectoris, myocardial infarction, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting, stroke or transient ischemic attack. Laboratory and physical assessment data, such as glycated hemoglobin (HbA1c), non-fasting lipid profile, serum creatinine, albuminuria (albumin-to-creatinine ratio), body mass index (BMI), and blood pressure were collected annually. Blood pressure was measured twice with a Welch Allyn Sphygmomanometer in the supine position after at least five minutes of rest. For each visit the mean blood pressure of two recordings was calculated.

Of the 1689 included patients, 1374 samples were eligible for further analyses to measure Prx4. Complete information on Prx4 and potential confounders in this patient group was available for 1161 patients.

Serum Peroxiredoxin 4

Prx4 levels were measured in serum samples collected at baseline and stored at -80°C until analysis in 2010. Because the performance of one freeze-thaw cycle has no consequences for assessing Prx4 levels, no influence of frozen storage on the assessed levels is to be expected. The validated immunoluminometric sandwich assay which measures Prx4 levels uses two monoclonal mouse antibodies both directed against amino acids 39 to 51 at the N-terminus of human Prx4, which excludes cross-reactivity with other members of the Prx family (19). The assay reports Prx4 concentration as arbitrary units per liter (U/L) and the functional assay sensitivity (interassay coefficient of variation <20%) is 0.51 arbitrary U/L. The limit of quantitation was 0.38 arbitrary U/L.

Clinical endpoints

The clinical endpoints were cardiovascular and all-cause mortality. In 2009, survival status and causes of death were obtained from the local hospital information system and the general practitioners concerning the ZODIAC cohort of 1998. Survival status and causes of death of the ZODIAC cohort of 2001 were obtained in 2005. Causes of death were coded according to the International Classification of Diseases, ninth revision (ICD-9).

Statistical analyses

SPSS version 16.0 (SAS Insitute, Cary, NC, USA) and STATA version 11 (StataCorp, College Station, Texas USA) were used for statistical analyses. Continuous variables are represented as mean (standard deviation - SD) for normally distributed values and as median (interquartile range - IQR) for non-normally distributed variables.

Cox proportional hazard models were used to investigate the association between Prx4 and (cardiovascular) mortality. The selected variables with possible confounding effects were age, gender, BMI, serum creatinine, smoking, diabetes duration, systolic blood pressure, cholesterol-HDL ratio, history of macrovascular complications, and albuminuria. Four models were chosen: a crude model including only Prx4 (model 1), a model with age and gender as additional confounders (model 2), a fully adjusted model (model 3), and finally a model that contained all the selected confounders except Prx4 (model 4).

Prx4 and serum creatinine were logarithmically transformed because of skewed distribution of the data.

The ph-test was used in combination with inspection of the Schoenfeld residuals to test the assumption of proportional hazards at baseline. Calibration was investigated using the Groennesby and Borgan test, assessing the goodness of fit and determining how well the predicted probabilities agree with the observed risk. When the average predicted risk matches the proportion that actually develops disease within subgroups of a prospective cohort, the model is considered well calibrated (24). In case of a significant association between Prx4 and (cardiovascular) mortality, the following analyses were performed. Harrell's C statistic, a rank-based measure, was used to compare how well the different models predict mortality (25). The higher the value the better the model predicts mortality. Furthermore, the integrated discrimination improvement (IDI) was calculated (26). The IDI is designed to evaluate the improvement in prediction of novel markers. It can be interpreted as the difference between model-based probabilities for events and non events for models with and without Prx4. The IDI is a global measure of correct reclassification regarding all possible cut-off values. The 95% confidence intervals for Harrell's C and IDI are given in the results.

Results

Baseline characteristics of the 1161 included patients are presented in strata according to Prx4 levels (Table 1). Median (interquartile range, IQR) serum level of Prx4 was 0.79 (0.53-1.25) arbitrary U/L. Patients with Prx4 above the median were older, had a higher BMI, lower eGFR, lower HDL cholesterol levels, higher HbA1c levels, higher prevalence of albuminuria and less frequently received lipid-lowering drugs, although the percentage of smokers was lower. After a median (IQR) follow-up of 5.8 (3.1– 10.1) years, 327 (28%) patients had died, of which 137 (42%) were attributable to cardiovascular disease.

Table 1. Baseline characteristics.

Baseline characteristic	Total n = 1161	Group 1 Prx4 < median*	Group 2 Prx4 > median	p-value
Age (years)	67 (12)	65 (11)	68 (11)	<0.001[1]
Gender male (%)	522 (45)	276 (48)	246 (42)	0.09[3]
Diabetes duration (years)	4.0 [2.0 – 9.0]	4.0 [2.0 – 9.0]	4.0 [2.0 – 9.0]	0.43[2]
eGFR (Cockcroft-Gault) (ml/min) [n=1022]	72 [57 – 92]	73 [58 – 94]	70 [55 – 90]	0.008[2]
BMI (kg/m^2)	28.7 [25.8 – 32.0]	28.1 [25.4 – 31.3]	29.2 [26.0 - 32.6]	<0.001[2]
Smoking (%)	220 (19)	125 (22)	95 (16)	0.03[3]
Systolic blood pressure (mmHg)	152 (24)	151 (24)	153 (24)	0.37[1]
HbA1c (%)	7.0 [6.2 - 8.1]	6.8 [6.2 – 7.9]	7.1 [6.3 – 8.2]	0.01[2]
Albuminuria (%)	455 (39)	182 (31)	273 (47)	<0.001[3]
Total cholesterol (mmol/l)	5.4 [4.8 – 6.2]	5.5 [4.8 – 6.2]	5.4 [4.7 – 6.2]	0.25[2]
HDL cholesterol (mmol/l)	1.2 [1.0 - 1.4]	1.2 [1.0 – 1.4]	1.1 [0.9 – 1.3]	0.003[2]
Cholesterol – HDL ratio	4.7 [3.8 – 5.8]	4.7 [3.8 – 5.6]	4.8 [3.9 – 6.0]	0.02[2]
Macrovascular complications (%)	413 (36)	195 (34)	218 (38)	0.16[3]
Receiving lipid-lowering drugs (%)	187 (16)	110 (19)	77 (13)	0.01[3]
Receiving antiplatelet therapy (%)	185 (16)	92 (16)	93 (16)	0.94[3]
Receiving ACE-I/ARB (%)	313 (27)	160 (28)	153 (26)	0.69[3]

Abbreviations: eGFR, estimated glomerular filtration rate; BMI, body mass index; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker. *) Median Prx4 (U/L): 0.79 [0.53 – 1.25]. Data are means (SD), medians [interquartile range], or n (%). P-values show (non)significance of group 2 compared to group 1. 1) Student's t-test. 2) Mann-Whitney U test. 3) Fisher's Exact test.

The survivors had lower median (IQR) baseline levels of Prx4 compared to the non-survivors [0.71 (0.48 – 1.05) arbitrary U/L] versus [1.05 (0.65 – 1.59) arbitrary U/L]; p<0.001. Increased levels of Prx4 were associated with higher rates of cardiovascular and all-cause mortality (Table 2). These associations persisted after adjustment for confounders in models 2 and 3.

Table 2. Hazard ratios for cardiovascular and all-cause mortality of the logarithmically transformed Prx4. Comparison of predictive capability of models for mortality risk prediction as determined by the Harrell's C statistic, and the IDI.

	Model 1	Model 2	Model 3	Model 4
Cardiovascular mortality				
Hazard ratio [95% CI]	1.93 [1.57 – 2.38]	1.75 [1.39 – 2.20]	1.63 [1.28 – 2.09]	n.a.
Harrell's C [95% CI]	0.65 [0.61 – 0.70]	0.77 [0.73 – 0.81]	0.82 [0.78 – 0.85]	0.81 [0.77 – 0.84]
IDI % [p]	n.a	1.97 [1.03 – 2.91]	0.97 [0.16 – 1.77]	n.a.
All-cause mortality				
Hazard ratio [95% CI]	1.73 [1.50 – 1.99]	1.50 [1.29 – 1.75]	1.44 [1.23 – 1.67]	n.a.
Harrell's C [95% CI]	0.64 [0.61 – 0.67]	0.79 [0.76 – 0.81]	0.81 [0.78 – 0.83]	0.80 [0.77 – 0.82]
IDI % [p]	n.a.	2.38 [1.41 – 3.34]	1.63 [0.82 – 2.44]	n.a.

Abbreviations: Prx4, peroxiredoxin 4; HR, hazard ratio; CI, confidence interval; IDI, integrated discrimination improvement; NA, not applicable. Cox regression models: Model 1: crude model with Prx4; Model 2: adjusted for age and gender; Model 3: adjusted for age, gender, smoking (dichotomous), body mass index, systolic blood pressure, duration of diabetes, serum creatinine level, cholesterol-HDL ratio, macrovascular complications (dichotomous), albuminuria (dichotomous); Model 4: all selected confounders without Prx4.

The Harrel's C values, as presented in Table 2, show that with increasing numbers of confounders, the better the model predicted cardiovascular mortality and all-cause mortality. However, no differences in the C values were observed between models 3 (the fully adjusted model) and model 4 (the fully adjusted model without Prx4).

Inspection of the Schoenfeld residuals and Stata's ph-test showed no violations of the assumption of proportional hazards. The goodness of fit analyses indicated that the models for cardiovascular mortality as well as for all-cause mortality were well calibrated.

Furthermore, the IDI was positive in models 2 and 3 for both cardiovascular and all-cause mortality.

Figures 1 and 2 show the estimated survival curves for cardiovascular and all-cause mortality according to patients with serum levels of Prx4 below the median compared to patients with serum levels Prx4 above the median – performed for model 3.

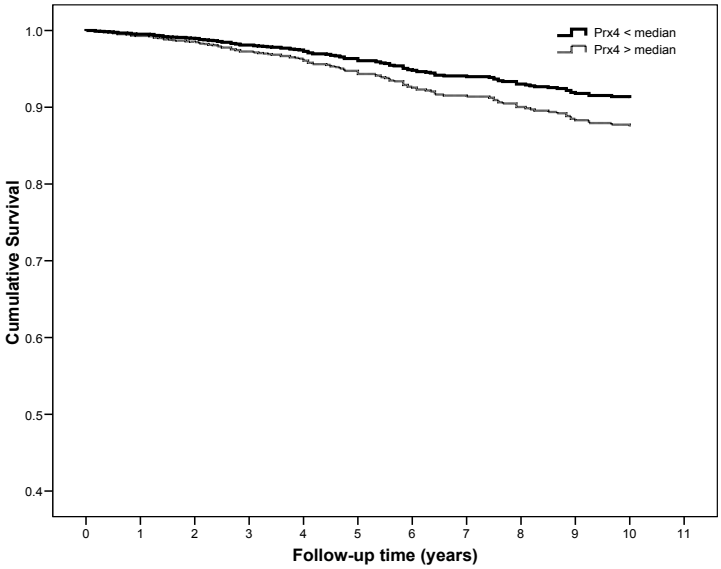

Figure 1. shows the estimated survival curves for cardiovascular mortality according to type 2 diabetes patients with serum levels of Prx4 below the median compared to patients with serum levels of Prx4 above the median – performed for model 3.

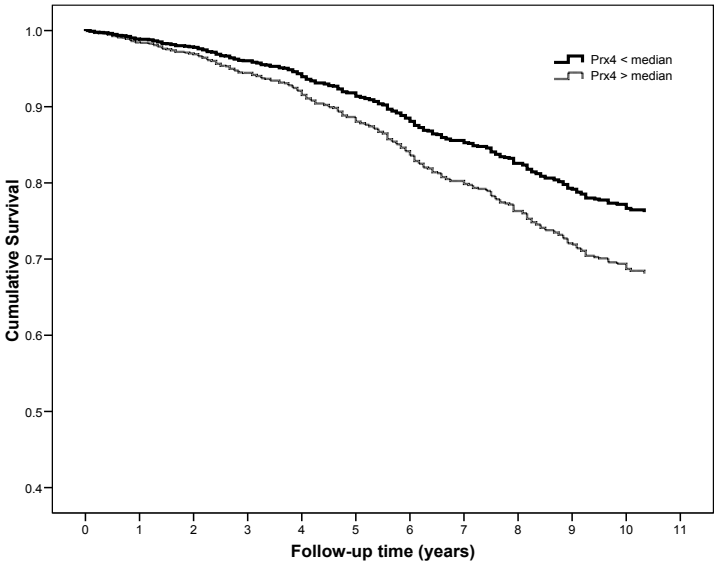

Figure 2 shows the estimated survival curves for all-cause mortality according to type 2 diabetes patients with serum levels of Prx4 below the median compared to patients with serum levels of Prx4 above the median – performed for model 3.

Discussion

Our study provides the first evidence that serum Prx4, a free serum antioxidant, is independently associated with increased risk for both cardiovascular and all-cause mortality in patients with T2DM. Prx4 may also have predictive ability and could potentially be used as a novel cardiovascular biomarker. When comparing the differences in Harrell's C between the models 2 and 4, adding Prx4 as a risk factor to age and gender (model 2) almost has the same predictive value as compared to all traditional cardiovascular riskfactors combined (model 3) for cardiovascular as well as for all-cause mortality. Although not completely proven, these results might point out a role of Prx4 in the prediction of (cardiovascular) mortality in T2DM.

Recently, the predictive capability of Prx4 was established for 30-days mortality in patients with nonspecific complaints presenting at the emergency department (27). Patients in this study cohort had a median age of 80 years, and Prx4 was not adjusted for possible confounders.

Higher circulating Prx4 levels were found in patients with inflammatory conditions like rheumatoid arthritis and sepsis, compared to healthy controls (19,21). In patients with sepsis the nonsurviving patients had higher Prx4 levels compared to the surviving patients and significant positive correlations were found between Prx4 and markers of infection and inflammation, like procalcitonin ($r = 0.61$, $p < 0.0001$), C-reactive protein ($r = 0.65$, $p < 0.0001$) and interleukin 6 ($r = 0.62$, $p < 0.0001$) (20,21). Correlations between Prx4 and antioxidative stress markers have also been described before, like albumin ($r = -0.54$, $p < 0.0001$) and bilirubin ($r = 0.37$, $p < 0.001$) (20). Additional information about correlations with other antioxidant markers and with oxidative damage markers would be useful to establish the use of Prx4 as a novel biomarker of oxidative stress.

In response to free oxygen radical production in T2DM, secretion of thiol-dependent peroxidases will increase to participate in the removal of these reactive oxygen species. It is hypothesized that the intracellular removal of hydrogen peroxides by Prx4 and secretion of the enzyme is proportionally upregulated in response to the surrounding oxidative stress (11,15). Oxidative stress also causes endothelial damage, which possibly could result in additional endothelial tissue leakage of Prx4,

contributing to even higher levels of serum Prx4. It still has to be investigated if Prx4 is actively removing hydrogen peroxides in the circulation. Perhaps, Prx4, being part of the antioxidant defense system, can rather be considered as a marker of endothelial cell damage and therefore would indirectly be linked to oxidative stress.

Patients with higher levels of Prx4 in our study revealed several characteristics that may have influenced Prx4 or oxidative stress in general. These include older age, higher prevalence of albuminuria, higher BMI, lower levels of HDL and less use of lipid lowering drugs like statins. However, even after adjustment for most of these confounders, the associations between Prx4 and mortality remained significant.

Our study also had a few limitations. Firstly, our analyses were performed in only 1161 out of the initially 1689 included patients. Secondly, because we only adjusted for a single baseline Prx4 value, we were not able to adjust for potential variability in Prx4 concentrations. Finally, care must be taken in interpreting the values of the IDI since this measure was not developed in the context of censored data. Besides this, there is no consensus regarding the interpretation of the magnitude of the IDI.

Strengths of our study were that it included over 1000 patients with a long follow up period and that we were able to include many confounders which were available for almost all patients. Another strength is the use of an immunoluminometric assay, which is a sensitive method for a reliable quantification of Prx4 in human serum (19).

To conclude, Prx4 is a circulating antioxidant and is independently associated with increased risk of cardiovascular and all-cause mortality in T2DM. Future studies are needed to answer the question whether Prx4, as a novel biomarker of oxidative stress, may be a new valuable cardiovascular predictor useful for risk stratification in T2DM.

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Disclosures

None

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CHAPTER 5

LIFE EXPECTANCY IN A LARGE COHORT OF TYPE 2 DIABETES PATIENTS TREATED IN PRIMARY CARE (ZODIAC-10)

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Abstract

Background Most longitudinal studies showed increased relative mortality in individuals with type 2 diabetes mellitus untill now. As a result of major changes in treatment regimes over the past years, with more stringent goals for metabolic control and cardiovascular risk management, improvement of life expectancy should be expected. In our study, we aimed to assess present-day life expectancy of type 2 diabetes patients in an ongoing cohort study.

Methodology and Principal Findings We included 973 primary care type 2 diabetes patients in a prospective cohort study, who were all participating in a shared care project in The Netherlands. Vital status was assessed from May 2001 till May 2007. Main outcome measurement was life expectancy assessed by transforming actual survival time to standardised survival time allowing adjustment for the baseline mortality rate of the general population.
At baseline, mean age was 66 years, mean HbA1c 7.0%. During a median follow-up of 5.4 years, 165 patients died (78 from cardiovascular causes), and 17 patients were lost to follow-up. There were no differences in life expectancy in subjects with type 2 diabetes compared to life expectancy in the general population. In multivariate Cox regression analyses, concentrating on the endpoints 'all-cause' and cardiovascular mortality, a history of cardiovascular disease: hazard ratio (HR) 1.71 (95% confidence interval (CI) 1.23 – 2.37), and HR 2.59 (95% CI 1.56 – 4.28); and albuminuria: HR 1.72 (95% CI 1.26 – 2.35), and HR 1.83 (95% CI 1.17 – 2.89), respectively, were significant predictors, whereas smoking, HbA1c, systolic blood pressure and diabetes duration were not.

Conclusions This study shows a normal life expectancy in a cohort of subjects with type 2 diabetes patients in primary care when compared to the general population. A history of cardiovascular disease and albuminuria, however, increased the risk of a reduction of life expectancy. These results show that, in a shared care environment, a normal life expectancy is achievable in type 2 diabetes patients.

Introduction

The incidence and prevalence of diabetes mellitus has risen worldwide during the past few decades. Recently published data from the Framingham Heart Study showed an absolute increase in the incidence of diabetes of ~ 2.5% yearly during the 1990s compared to the 1970s (1). The proportion of cardiovascular disease attributable to diabetes mellitus has increased as well (2). Other studies over the last decades of the previous century also showed higher mortality rates in diabetes mellitus compared to the general population, mostly due to cardiovascular events (3-7). However, since the progress in effective pharmaceutical interventions and more stringent regimens for the treatment of hyperglycemia, hypertension, dyslipidemia, and other cardiovascular risk factors, trends towards a reduction of (cardiovascular) mortality rates amongst diabetic patients have been reported (8-12). This improvement of survival was hoped to eventually be comparable to the decrease in cardiovascular mortality rates in the general population thanks to aggressive management of cardiovascular risk factors.

Most published data were extracted from representative national cohorts in North-America or the United Kingdom. Some reports showed a decline in mortality rates amongst diabetic men only, whereas women showed an increase or no change in mortality rates at all (4,8,11,12). A recently published Scandinavian study showed a substantial decrease in mortality rates from coronary heart disease in all age groups irrespective of sex and diabetes status over two consecutive time periods. However, the more than twofold higher mortality from coronary heart disease in diabetes patients compared to the non-diabetic population remained over time. Still, these findings suggested a longer survival in diabetes patients resulting from intensified treatment of cardiovascular risk factors (13).

Our aim was to investigate present-day life expectancy in a type 2 diabetes population treated in primary care, with additional support both for patients and health care professionals in a shared care setting in a Western European country.

Methods

Participants

In 2001, 973 type 2 diabetes patients participated in a cross-sectional study with measurements of skin advanced glycation endproduct (AGE) accumulation as described previously (14). This study was embedded in a long-term shared care project (ZODIAC: Zwolle Outpatient Diabetes project Integrating Available Care) concerning a primary care treated population-based sample of type 2 diabetes patients in an eastern district of The Netherlands. Figure 1 shows an overview of the enrolment of the current study cohort started from the beginning of the ZODIAC. During this project, 32 general practitioners (GPs) were supported by hospital diabetes specialist nurses and consultant-physicians (15).

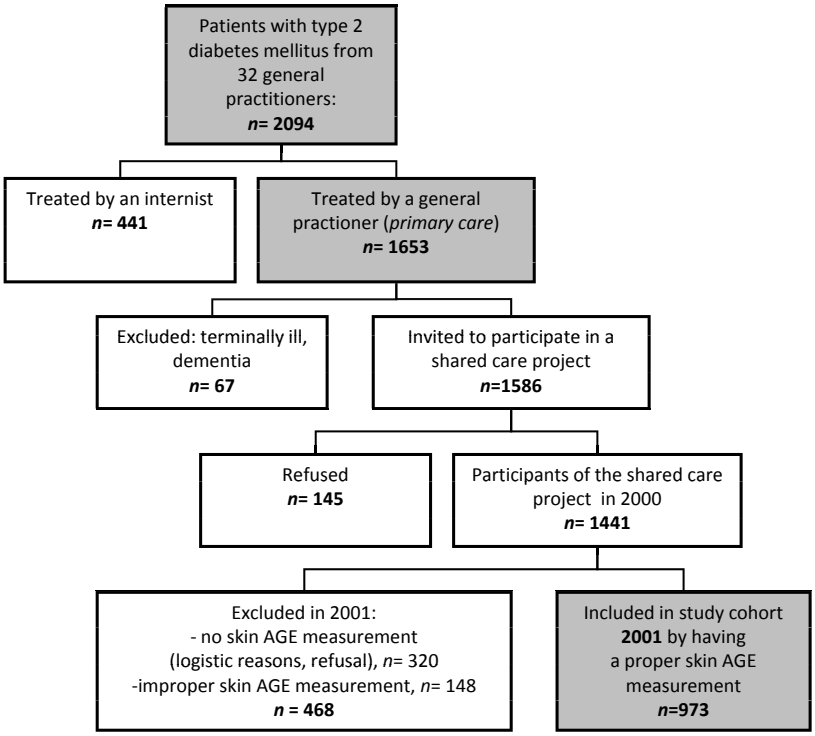

Figure 1. Flowchart of the enrolment of the type 2 diabetes study cohort from 32 general practitioners of a district in The Netherlands.

In short, all type 2 diabetes patients were exclusively treated by their GPs and visited the diabetes specialist nurses for evaluation of metabolic control and diabetes related complications annually. After these evaluations, treatment advice for individual patients as well as for benchmarking was given to the GPs by internists in the Isala Clinics in Zwolle, the Netherlands. Advices and referrals were based on guidelines of the Dutch College of General Practitioners (16).
Patients with a cognitive disability or terminal disease were not included in the ZODIAC study because of their inability to undergo educational programs. Furthermore, patients who were physically unable to visit the diabetes specialist nurse at the outpatient clinic were not enclosed in the present cohort.

Ethics Statement
This study was approved by the local ethical committee of the Isala Clinics, Zwolle, The Netherlands and all patients gave written informed consent.

Description of Procedures
Methods of clinical data collection and laboratory assessments have been described in detail elsewhere (14). Before participation in our study, based on the 1997 ADA guidelines and the Dutch primary care standard (16), diagnosis of diabetes mellitus was already made in individuals with fasting plasma glucose levels ≥ 7.0 mmol/liter and the following definitions representing diabetic complications at baseline were: retinopathy, which was defined as the presence of at least background retinopathy or a history of laser coagulation for diabetic retinopathy. Albuminuria was defined as an albumin-to-creatinine ratio >3.5 mg/mmol for women or >2.5 mg/mmol for men. Diminished sensibility at least at one foot was considered as neuropathy, tested with a 5.07 Semmes-Weinstein monofilament, applied on three areas of each foot. The presence of microvascular disease was defined as meeting the criteria of retinopathy, albuminuria, and/or neuropathy. The presence of cardiovascular disease at baseline was defined when meeting at least one aspect of cardiovascular disease: ischemic heart disease (IHD), International Classification of Diseases ninth revision (ICD-9), codes 410-414 and/or a history of coronary artery bypass surgery or percutaneous coronary intervention, cerebrovascular accidents including transient ischemic attacks

(CVAs / TIAs) and/or peripheral vascular disease (PVD). PVD was defined as surgical intervention, history of claudication and/or absent pulsations of ankle or foot arteries (absence of pulsations of the dorsalis pedis arteries bilaterally was not scored as PVD when tibial posterior artery pulsations were present).
Mortality was registered from the date of inclusion until May 2007. Death was certified according to the following procedure. In addition to the list of deceased patients reported in the files of the scheduled annual follow-up visits, survival status of the patients was obtained from the local hospital information system and verified with the GPs. Date of death was collected likewise. None of the GPs had involvement or interest in study outcome. Causes of death were coded according to ICD – 9 and categorised as: neoplasms (140 – 239), diseases of the cardiovascular system (390 – 459), diseases of the respiratory system (460 – 519), diseases of the digestive system (520 – 579), diseases of the genitourinary system (580 – 629), injury and poisoning (800 – 999). Sudden death, with symptoms present for less than one hour, was encoded in the category of coronary heart disease. For the in-hospital deaths, the medical records were retrieved. For the out-of-hospital deceased patients, the assigned causes of death by the GPs were obtained from the medical records of the GPs. The coded causes of death were combined to all-cause mortality (all codes) and cardiovascular mortality (390 – 459 or sudden death).

Statistical Methods

Life expectancy analysis was performed primarily by the use of 'standardised survival time' (SST), which is a novel approach to survival analysis (17). SST is another expression of follow-up time than survival time in years. This method provides survival time, which is adjusted for the median residual life span of individuals in the general population with the same age and sex. Due to this standardisation of survival time there is no influence of the interactions between age and the presence or effects of other risk factors due to age. Furthermore, it allows assessment of the effectiveness of treatment on regaining a normal residual life span. SST was calculated as the ratio between the observed survival time (follow-up time) of an individual and the median residual life span of individuals with the same age and sex in the general population at the starting date of the study. The median residual life span was derived from

gender specific reports provided by the Dutch Central Office of Statistics, which is the national Dutch institution of statistics and demographics (18). The SST at baseline is defined as 0, and a SST ratio of 1 is defined as follow-up time corresponding with the life expectancy of the general population. Direct comparisons between study samples and the general population were done by comparing the 95% confidence interval (CI) of each median SST with an expected value of 1. The 95% CI of mortality at a SST of 0.25 and 0.5 were calculated and compared with the expected mortality as calculated for the age and gender matched general population, assuming Poisson distribution of the events. Kaplan-Meier curves were constructed for survival and for standardised survival. A Cox proportional hazard model to estimate hazard ratios (HR) and 95% CI was used in the standard way using survival time in years, and additionally by using SST. Methodologically, it is allowed to use SST instead of survival time in years in a Cox-regression model, as it is consistent with the preconditions of a Cox regression analysis: an increase in mortality and an increase in follow-up time have to be present. This new statistical approach underlines the prognostic value of the mentioned risk factors, irrespective of age and sex. Eliminating the effects of age and sex excludes the influence of disease-specific risk of age and sex in the standardised analysis. P values <0.05 were considered to be statistically significant. Clinical and laboratory variables with an expected effect on mortality risk were first analysed in a univariate analysis, and secondly, in a multivariate model. Detailed analyses were performed specifically for two end-points: all-cause mortality and cardiovascular mortality.

Results

Characteristics of the 973 type 2 diabetes patients at baseline (2001) are shown in Table 1. The population had a relatively short median diabetes duration of 4.2 (interquartile range 1.6 – 8.3) years and on average an acceptable to good glycemic control (mean HbA1c 7.0%). Table 1 also shows the baseline characteristics of patients when subdivided in survivors (791 patients) and non-survivors. At the end of a median follow-up duration of 5.4 years (interquartile range 5.1 – 5.6), 165 patients

had died (17%); 17 patients were lost to follow-up. Minimum follow-up duration of all survivors was 5.0 years. Ten of the lost to follow-up persons had a last visit to the outpatient clinic between baseline and the end of follow-up; this last registered visit date was defined as the end of follow-up for these patients. The remaining 7 persons lost to follow-up had a mean age of 68 years, were non-smokers, and 2 patients had cardiovascular disease at baseline. Their median diabetes duration was 9.7 years with a mean HbA1c of 7.5%.
The proportion of prescribed lipid-lowering drugs, renin-angiotensin system (RAS) inhibitors and antiplatelet therapy at baseline, is shown at the end of Table 1.

At baseline, antiplatelet drugs were significantly more prescribed in the non-survivors compared to the survivors. The proportion of cardiovascular deaths in the study population (47%) was increased compared to the general population. In 2007, 31% of all deaths in the general Dutch population were due to cardiovascular disease, with a highest relative incidence of 38% cardiovascular deaths in the population above 85 years (18).
Figure 2 shows the Kaplan-Meier curve of the cumulative proportion of survivors in our type 2 diabetes population against survival time in years. A Kaplan-Meier plot of the cumulative proportion of deaths in our study population against standardised survival time is shown in Figure 3; the expected mortality for the age- and gender-matched general population is also shown. The median standardised survival time in our study population was 1.00 [95% confidence interval (CI) 0.88 – 1.12] and did not differ from the general population. The cumulative proportion of deaths at half standardised survival time (SST = 0.50) was 0.20 (95% CI 0.16 – 0.23), which again did not differ from the expected value of 0.18 in the general population.

Table 1. Baseline characteristics of type 2 diabetes patients: total and subdivided in survivors and non-survivors, expressed as mean ± SD or *n* (%).

Characteristic	Total (N = 973)	Survivors (N=791)	Non-survivors (N=165)	p-value
Age in years	66.4 (11.3)	64.8 (11.1)	73.6 (9.4)	<0.001
Male gender (%)	47	46.3	50.9	0.278
Smoking (%)	19.3	19.3	20.6	0.71
Body mass index (kg/m^2)	29.38 (4.87)	29.5 (4.8)	28.7 (5.1)	0.077
Systolic blood pressure (mmHg)	146.01 (20.15)	145 (20)	149 (20)	0.015
Diastolic blood pressure (mmHg)	81.18 (10.34)	81 (10)	80 (11)	0.046
Diabetes duration (years)	[a]4.16 (1.62-8.31)	[a]3.92 (1.50-8.04)	[a]5.03 (2.40-10.8)	0.002
HbA1c (%)	6.96 (1.3)	6.95 (1.32)	6.998 (1.23)	0.69
Creatinine (µmol/l)	96.0 (19.88)	94.56 (17.6)	103.16 (27.69)	<0.001
Creatinine clearance (ml/min)	76.13 (26.91)	78.8 (26.6)	63.22 (24.75)	<0.001
Urinary albumin-to-creatinine ratio (mg/mmol)	[a]1.49 (0.80-4.17)	[a]1.35 (0.75-3.44)	[a]3.09 (1.23-11.01)	0.001
Total cholesterol (mmol/l)	5.16 (1.01)	5.17 (1.02)	5.08 (1.00)	0.302
Cholesterol-to-HDL ratio	4.34 (1.23)	4.37 (1.21)	4.22 (1.36)	0.171
HDL cholesterol (mmol/l)	1.25 (0.33)	1.24 (0.32)	1.29 (0.35)	0.141
LDL cholesterol (mmol/l)	2.87 (0.93)	2.85 (0.92)	2.92 (0.98)	0.388
Triglycerides (mmol/l)	2.32 (1.36)	2.39 (1.40)	2.03 (1.14)	0.002
Microvascular disease (%)	54.1	50.2	70.9	<0.001
Retinopathy (%)	19.6	18.5	24.8	0.050
Microalbuminuria (%)	25.5	21.4	45.5	<0.001
Neuropathy (%)	29.1	26	40.6	<0.001
Cardiovascular disease (%)	39.5	34.6	63.6	<0.001
Ischemic heart disease (%)	21.5	19.6	30.3	0.002
Cerebrovascular disease (%)	7.8	6.4	14.5	<0.001
Peripheral vascular disease (%)	23.0	18.1	47.3	<0.001
RAS-inhibitors[b] (%)	37.2	36.5	39.4	0.489
Lipid-lowering drugs[c] (%)	29.8	30.7	26.1	0.234
Antiplatelet drugs (%)	24.9	22.1	38.2	<0.001
Diabetes treatment – Diet only (%)	20.2	21.4	16.4	
Oral medication (%)	64.1	64.3	63	
Insulin (%)	9.8	8.3	15.8	
Both (%)	5.9	5.9	4.8	

Seven patients were lost to follow-up and did not define the baseline characteristics of the survivors/non-survivors.

[a]Median and interquartile range, [b]Angiotensin-converting enzyme inhibitors and Angiotensin II receptor blockers, [c]Large majority represented by statins (99%). Reference values of the laboratory: HbA1c 4.0-6.0 %, creatinine 70-110 µmol/l, creatinine clearance (Cockcroft-formula) 80-120 ml/min, urinary albumin-to-creatinine ratio 0-2.5 for men and 0-3.5 for women, total cholesterol 3.5-5.0 mmol/l.

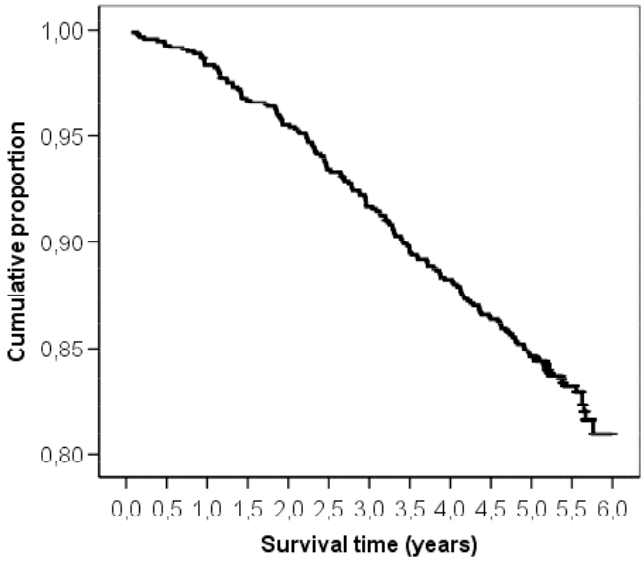

Figure 2. Kaplan-Meier survival curve for survival in years in the entire type 2 diabetes group.

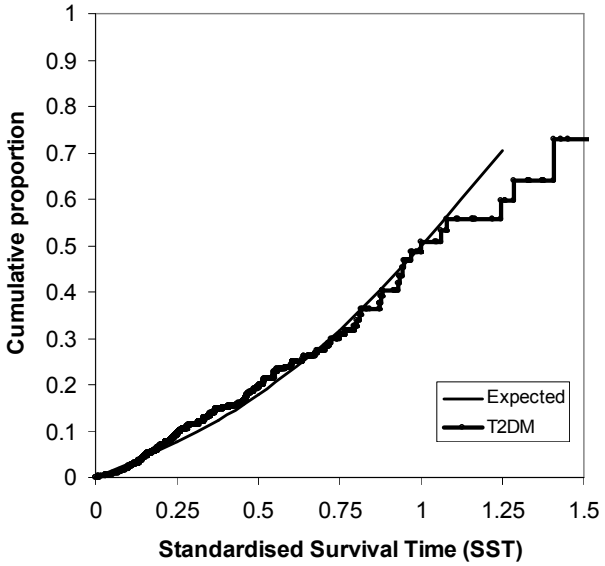

Figure 3. Kaplan-Meier plot of the cumulative proportions of deaths against Standardised Survival Time (SST) in the entire type 2 diabetes group. The median SST in type 2 diabetes mellitus (T2DM) 1.00 is not different from the general Dutch population 1.00 (Expected); the observed mortality (all-cause) at SST 0.25 and 0.50 of 0.09, respectively 0.20 does not significantly differ from the general Dutch population (0.08 respectively 0.18, p>0.1).

Figure 4 shows the mortality rate in type 2 diabetes patients with albuminuria at SST 0.25 of 0.15 (95% CI 0.10 – 0.19), which was higher than the expected value of 0.076 (p=0.002). At SST 0.50 mortality rate was 0.26 (95% CI 0.19 – 0.33), which was also higher than the expected value of 0.18 (p=0.014). This also proved to be the case for type 2 diabetes patients with a history of cardiovascular disease, who had a higher mortality at SST 0.25 [0.13 (95% CI 0.096 – 0.17), p<0.001] and at SST 0.50 [0.25 (95% CI 0.19 – 0.30), p<0.0001], Figure 5.

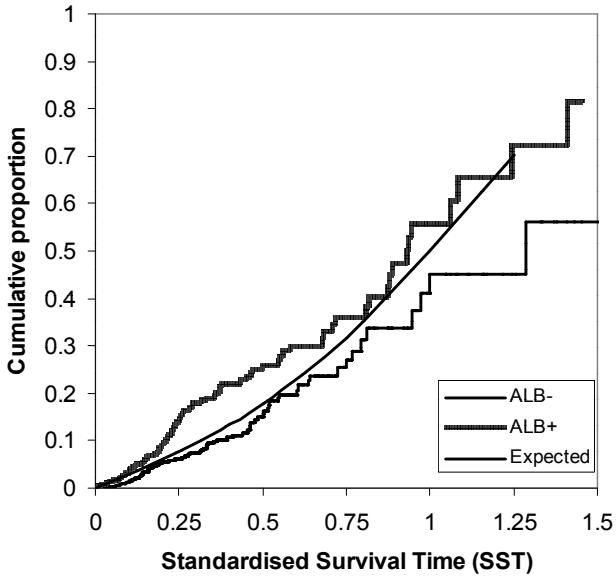

Figure 4. Kaplan-Meier plot of the cumulative proportions of deaths in patients with albuminuria against Standardised Survival Time. Cumulative proportions of deaths (all causes) against Standardised Survival Time (SST) in type 2 diabetes patients with albuminuria (Alb) yes/no (+/-), compared to the expected deaths of the general population. Differences in mortality between the type 2 diabetes-subgroups and the general population are tested at SST= 0.25 and SST=0.5. Mortality rate at SST 0.25 is 0.15 [95 % confidence interval (CI) 0.10 – 0.19] and the expected value is 0.076 (p=0.002). At SST 0.50 mortality rate is 0.26 (95 % CI 0.19 – 0.33), which was also higher than the expected value of 0.18 (p=0.014).

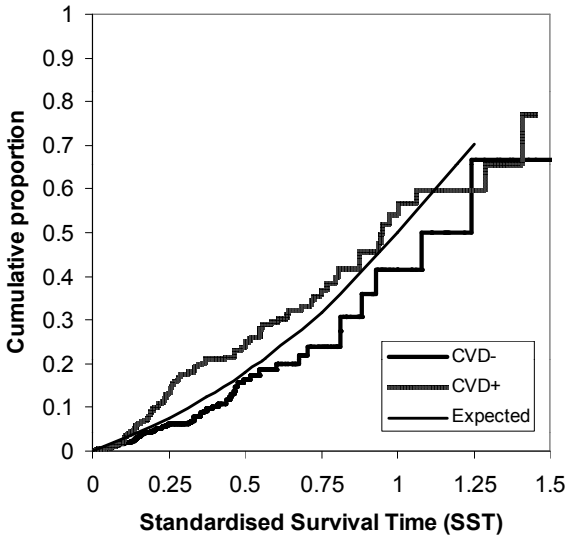

Figure 5. Kaplan-Meier plot of the cumulative proportions of deaths in patients with previous cardiovascular disease against Standardised Survival Time. Cumulative proportions of deaths (all causes) against Standardised Survival Time (SST) in type 2 diabetes patients with previous cardiovascular disease (CVD) yes/no (+/-), compared to the expected deaths of the general population. Differences in mortality between the type 2 diabetes-subgroups and the general population are tested at SST= 0.25 and SST=0.5. Mortality rate at SST 0.25 is 0.13 (95% CI 0.096 – 0.17) and an expected value is 0.076, $p<0.001$. At SST 0.50 mortality rate is 0.25 (95% CI 0.19 – 0.30) and the expected value is 0.18, $p<0.0001$.

Table 2 shows the hazard ratios (HRs) and 95% CI of univariate and multivariate Cox-regression analyses for all-cause mortality. The HRs in the univariate analyses are higher for all cardiovascular disease items compared to the method of using SST in the model. In the multivariate analysis, predictive factors for all-cause mortality were comparable for both methods when age and gender were included in the model of the standard method: a history of cardiovascular disease (HR 1.79 and 1.71) and, albuminuria (HR 1.79 and 1.72). Univariate analysis of the endpoint: cardiovascular mortality (not shown in Table 2) resulted in the same significant predictive factors, but with higher HRs.

Multivariate analysis of cardiovascular mortality resulted in the same significant predictive factors with higher HRs (SST) as well: albuminuria 1.83 (95% CI 1.17 – 2.89); history of cardiovascular disease 2.59 (95% CI 1.56 – 4.28). Smoking, systolic blood pressure, diabetes duration and HbA1c did not reach significance in both multivariate models.

Table 2. Predictors of overall mortality in type 2 diabetes mellitus by univariate and multivariate Cox regression analysis using "survival in years" (= standard method) and using "standardised survival time".

Predictors of all-cause mortality	Survival in years			Standardised survival time [a]		
	Univariate					
	HR	**95% CI**	***p-value***	**HR**	**95% CI**	***p-value***
Gender (man = reference)	0.84	0.62 – 1.14	0.25	0.91	0.67 – 1.24	0.56
Age	1.08	1.06 – 1.10	<0.001	1.00	0.98 – 1.02	0.90
Smoking	1.10	0.75 – 1.60	0.63	1.49	1.02 – 2.18	0.039
Systolic blood pressure	1.01	1.00 – 1.02	0.016	1.00	0.99 – 1.01	0.66
Diabetes duration	1.03	1.02 – 1.05	0.001	1.02	1.00 – 1.04	0.073
HbA1c	1.02	0.91 – 1.15	0.71	1.08	0.95 – 1.22	0.23
Albuminuria (yes/no)	2.33	1.71 – 3.17	<0.001	1.81	1.32 – 2.46	<0.001
History of cardiovascular disease (yes/no)	2.95	2.15 – 4.06	<0.001	1.87	1.35 – 2.58	<0.001
Use of lipid-lowering drugs (yes/no)	0.83	0.59 – 1.18	0.30	1.34	0.80 – 1.62	0.48
Use of antiplatelet drugs (yes/no)	2.00	1.46 – 2.74	<0.001	1.47	1.07 – 2.01	0.018
	Multivariate					
Gender (man = reference)			NS	x	x	x
Age	1.07	1.05 – 1.09	<0.001	x	x	x
Smoking (yes/no)			NS			NS
Systolic bloodpressure			NS			NS
Diabetes duration			NS			NS
HbA1c			NS			NS
Albuminuria (yes/no)	1.79	1.30 – 2.46	<0.001	1.72	1.26 – 2.35	0.001
History of cardiovascular disease (yes/no)	1.79	1.29 – 2.50	0.001	1.71	1.23 – 2.37	0.001
Use of lipid-lowering drugs (yes/no)			NS			NS
Use of antiplatelet drugs (yes/no)			NS			NS

Abbreviations: HR, hazard ratio; CI, confidence interval; NS, not significant. [a] The standardised survival time was calculated as the ratio between the observed survival time of an individual and the median residual life span of individuals with the same age in the general population.

Discussion

This study shows a normal median overall life expectancy in a defined cohort of type 2 diabetes patients treated in a primary care setting, during a follow-up period from 2001 till 2007. This finding strongly suggests that current available treatment strategies may eventually lead to a life expectancy equal to the general population in this subset of type 2 diabetes patients. Secondly, in this type 2 diabetes study population, patients with a history of cardiovascular disease and/or the presence of albuminuria still had an increased risk to die before their median life expectancy was reached. The differences in effects of all items of cardiovascular disease on 'survival in years' and SST, could be explained by the age correction enclosed in the SST – method. As the prevalence of cardiovascular diseases is increasing with increasing age, SST does have definite advantages compared to the 'classical survival time' in identifying premature mortality.

Finally, we still found an increased proportion of deaths due to cardiovascular disease compared to the general population (47% versus 31%). This is in agreement with established observations of increased cardiovascular disease in diabetes, and also with the fact that the presence of classical cardiovascular risk factors still is most intimately related to life expectancy reduction (13,19).

The United Kingdom Prospective Diabetes Study reported a 5 years reduction of life expectancy for males aged 45 to 50 years at diagnosis of diabetes when compared to the general United Kingdom population (6). Estimations of reduction of life expectancy for patients with diabetes diagnosed at an older age are not presented explicitly in this paper, but might be smaller than 5 years, as other studies showed that reduction of life expectancy decreases with diagnosis at older age (5,12,20).

A large study of the non institutionalised United States population, which was conducted between 1971 and 1993, showed a median reduced life expectancy of 8 years for the diabetic population aged 55-64 years, and a 4 years reduction for the diabetic population aged 65-74 years (4). However, these studies were all executed in a period during which treatment with statins, angiotensin-converting enzyme inhibitors and angiotensin-1 receptor blockers, and antiplatelet medication was much less common practice. A more recent study, showing slightly increased mortality in

women but no excess mortality in men, included exclusively patients diagnosed with type 2 diabetes mellitus over the age of 65 (12). Our study is of additional value, as we included primary care type 2 diabetes patients of all ages, representing a large amount of the type 2 diabetes patients in The Netherlands, where the majority of subjects with type 2 diabetes is treated in primary care according to national guidelines. Sixty-four percent of our study population was diagnosed with type 2 diabetes mellitus before the age of 65 years.

A previous study in the first ZODIAC-cohort (1998) reported an annual mortality rate of 4.8% between 1998 and 2000 (the first three years of the shared care project), definitely higher than the mortality rate in the present analysis (~3 %), which was performed over the subsequent years within this shared care environment (21). This difference could be explained by the fact that the earlier analysis was performed in a more extended type 2 diabetes cohort, which also included patients who were referred to secondary care. It is also possible that the cohort as presented in this first analysis had yet to benefit from longer term participation in a shared-care environment with supportive care and monitoring of implementation of the guidelines.

More than half of our population received either a statin, RAS-inhibitor or aspirin at baseline. At follow-up, this proportion had increased to at least 80%. Widespread treatment of the traditional cardiovascular risk factors resulted in vastly improved blood pressure readings and lipid levels. This could also be the explanation for disappearance of systolic blood pressure from the model to predict mortality. Recent studies, although maybe underpowered, addressed the importance of statins and blood pressure lowering drugs in patients with type 2 diabetes mellitus, showing a reduction in cardiovascular events with these lipid-lowering drugs compared to placebo (22-24).

HbA1c had also no effect on life expectancy in uni- and multivariate analysis. This may possibly be explained by the low number of patients with poor glycemic control (only 7% had a HbA1c >9%). Alternatively, other mechanisms could be involved in the development of diabetes related complications. E.g., we recently reported increased levels of advanced glycation endproducts (AGEs) rather than HbA1c in the same study group, to be related to chronic complications (14).

There are some limitations to our study regarding the possible general applicability of the results. Diabetic patients who were referred to the secondary care in the past, mainly for reasons of poor metabolic control or comorbidity, were not included in this study and almost certainly will have a reduced life expectancy. Also, there has been a selection bias by excluding diabetic patients with a very short life expectancy (terminally ill patients, cognitive disabled people and patients who were unable to undergo educational programs), as described in the methods section. Still, the selection comprised a considerable subset of the total population known with type 2 diabetes (see figure 1), and 40% of the included study population were known with cardiovascular disease at baseline.

Despite this apparent selection bias, we still are able to conclude that we defined a large subset of patients with a life expectancy comparable to that of the general population of the same age and sex. In The Netherlands, the large majority (70 – 80%) of type 2 diabetes patients is treated in primary care or in a shared-care setting. Therefore, this study population could be representative for the majority of type 2 diabetes patients in The Netherlands, and probably also for a larger part of type 2 diabetes patients in other countries with structured diabetes care.

Our choice to compare life expectancy of this type 2 diabetes cohort to the general population can be criticised, since the general population also includes people with diabetes, cardiovascular disease, cancer, and other life shortening diseases. We nevertheless preferred to choose the general population instead of a non-diabetic control group, since one of the aims of caregivers in medical practice is to regain a life expectancy for their patients equal to the general population when life expectancy is reduced due to a specific disease.

To visualise whether a life expectancy equal to the general population had been achieved, we used SST. Traditional survival analysis focuses more on ‘mortality’ within a certain follow-up time, but with this more conventional method it is not clear whether it is ‘normal mortality’ or ‘excess mortality’. Using SST, the mortality rate is adjusted for the median survival of subjects in the general population of the same age and sex. In this way, we eliminate the effect of age and sex, by excluding the influence of disease-specific risk of age and sex in the standardised analysis.

Excess mortality or a reduced life expectancy will be identified more easily in that way. We consider the results of this study to be relevant for clinical practice, because they offer a hopeful perspective of a definitely improved life expectancy in type 2 diabetes patients. We suggest that those results are also (partly) due to the fact that these patients were and are participating in a care system promoting adherence to evidence-based guidelines and to a system emphasizing close cooperation between health care providers focusing on this patient group.

In summary, this study shows a normal life expectancy in a large subset of type 2 diabetes patients treated in a primary care setting compared to the general population. The presence of previous cardiovascular disease and albuminuria, however, is still associated with a markedly reduced life expectancy.

Funding sources

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PART II

SKIN AUTOFLUORESCENCE IN CHRONIC KIDNEY DISEASE AND END STAGE RENAL DISEASE

CHAPTER 6

AGES, AUTOFLUORESCENCE AND RENAL FUNCTION

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Nephrology Dialysis Transplantation 2008, 25: 1 – 4

Introduction

Accelerated formation and accumulation of AGEs occur under circumstances of hyperglycemic or oxidative stress in age-related and chronic diseases like diabetes mellitus, chronic renal failure , neurodegenerative diseases, osteoarthritis, and non-diabetic atherosclerosis (1 – 5). Accumulation of irreversibly formed and chemically stable AGEs occurs on long-lived proteins such as collagen in the skin, but also in vascular basement membranes. This affects their structure and function resulting in vascular damage. Adequate renal clearance capacity is an important factor in the effective removal of AGEs. In renal failure there is a profound decrease in clearance of AGE free adducts, which are formed mainly from proteolysis of glycated proteins. Plasma levels of these products are up to 40 fold higher in hemodialysis patients compared to healthy subjects. Increased levels of AGE free adducts in plasma is also a characteristic of acute and chronic renal failure, whereas accumulation of AGE residues on plasma proteins appears to be limited to chronic renal failure (6 – 8). Generally, AGE residues on plasma proteins are not decreased during a dialysis session, while AGE free adducts are indeed removed by hemodiafiltration or other dialysis procedures (7). Little is known about tissue accumulation of AGEs on long-lived proteins in patients with chronic renal failure and patients on hemodialysis.
Furthermore, both uraemic toxicity and some modalities of renal replacement therapy contribute to increased oxidative stress, inducing protein modification, which either directly or indirectly contribute to the increased formation of AGEs (2,9). Since tissue accumulation of AGEs on long-lived proteins is a long-term process, quantitation of the collagen-bound AGEs could reflect “metabolic memory” over several years. Up till recently, skin biopsies were needed to properly assess the level of tissue AGE accumulation; as this is an invasive and time-intensive method, it is not feasible in daily practice.

The autofluorescence reader

The application of a newly developed noninvasive device, the autofluorescence reader (AFR), gives the opportunity to measure skin autofluorescence (AF), which has shown to be reasonably well correlated with skin content of some AGE residues.

The technique has been validated against AGE measurements in skin biopsies from the site of the skin AF measurement, taken in patients on hemodialysis, patients with diabetes mellitus and healthy controls. Skin AF correlates with skin levels of some AGE residues: N^{ε}-carboxymethyl-lysine (CML), $r = 0.55$ ($p < 0.001$); pentosidine, $r = 0.55$ ($p < 0.001$); N^{ε}-carboxyethyl-lysine (CEL), $r = 0.47$ ($p = 0.002$) (10,11) . Thus, compared to this laborious and time intensive invasive technique and to serum or plasma AGE measurements, the AFR allows for a noninvasive and complete automated measurement within 30 seconds with immediate presentation of the result, which makes this device suitable for clinical application. The AFR illuminates the volar side of a skin surface of the arm of ~4 cm^2, guarded against surrounding light (Figure 1a).

Figure 1a. Autofluorescence reader in a clinical setting

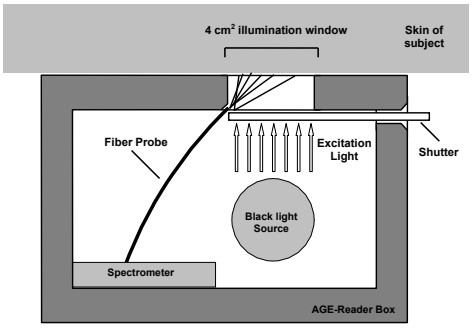

Figure 1b. Schematic view of the autofluorescence reader

The principle of skin AF is based on the fluorescent properties of certain AGEs. The excitation light source has a peak intensity of ~370 nm, and emission light and reflected light from the skin is measured with a spectrometer in the range 300 – 600 nm (Figure 1b). Skin AF is calculated by dividing the average light intensity of the emission spectrum by the average light intensity of the excitation spectrum, and is expressed in arbitrary units. Skin reflection is taken into account by using an internal reflection standard. Managing the instrument does not require special training or skills, and needs no special preparation of the subjects. Reproducibility of the device has been tested in different study populations and showed a mean relative error in skin AF of ~ 5%. Mean age-corrected skin AF per measuring month, per examiner,

and per AFR-system did not differ significantly as well (12). An important limitation of the original AFR was the inability to measure people with dark skin type, because of the high absorption grade of the excitated light. Recent developments of the AFR device have reduced this limitation to a more limited range of people with a very dark skin type. Other limitations of skin AF measurement: non-fluorescent AGEs are not detected – by definition – with skin autofluorescence, while the fluorescence of other non-AGE tissue components in the same range of wavelength may act like confounders. Not all AGEs show fluorescent characteristics: hydroimidazolones, CML and CEL are important AGEs, but not fluorescent. Furthermore, as already mentioned, the fluorescence patterns used by the AFR are not specific for fluorescent AGEs only, but could also be due to other fluorophores like nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD). There could also be a contribution of the fluorescent oxidation adduct N-formyl-kynurenine (13,14).
In validation studies, skin AF correlated with the specific AGE skin levels of pentosidine, CML, and CEL. Both fluorescent and non-fluorescent skin AGE levels correlated with each other, as indicated by the correlation between pentosidine and CML ($r = 0.46$; $p < 0.001$) (10,11). Despite the reasonable correlation between skin AF and skin biopsy AGE content, results still have to be interpreted with the mentioned limitations and pitfalls in mind, and perhaps more detailed validation of this technique is required.

Clinical evidence

Skin AF has already been shown to be related to age, smoking, and diabetes mellitus. In type 2 diabetes patients, skin AF is related to HbA1c, diabetes duration, microalbuminuria and diabetic complications (12,15). Skin AF has also shown its predictive value for mortality and the development of microvascular disease in type 2 diabetes mellitus, as well as for mortality in hemodialysis patients (16 – 19). Cross-sectional and longitudinal studies, measuring skin AF, are ongoing in other patient groups, in order to find out whether skin AF is a predictor for the development of morbidity or mortality in those patient groups as well.

AGEs and renal disease

Besides hyperglycemia and increased oxidative stress, decreases in glomerular filtration rate (GFR) appear to be an important determinant contributing to the accumulation of AGEs. As already mentioned, a decline in renal function leads to elevated serum AGE levels. Such a relationship was confirmed in diabetes mellitus, where progressive nephropathy with decreased renal function was associated with increased accumulation of AGEs (20).

Skin AF in renal failure is not only associated with, but also strongly predictive for cardiovascular disease, as shown by the independent predictive value of skin AF for total and cardiovascular mortality in hemodialysis patients (19). As for cardiovascular dysfunction in hemodialysis patients, it has been shown that plasma AGEs were not associated with diastolic function, while skin AF was independently associated with diastolic function (21). Prevention of the decline in renal function will probably lead to a reduced accumulation rate of AGEs, which in turn might contribute to a less dire cardiovascular outcome in patients with renal disease. The relative importance of this factor compared to other already known risk factors still awaits proper assessment, however.

Conventional methods of renal replacement therapy are only partially effective with regard to AGE clearance; the degree of removal is also dependent on the frequency and duration of dialysis (22,23). Also, treatment itself may contribute to AGE accumulation; oxidative stress is an important factor leading to AGE-formation, and some hemodialysis membranes – depending on their degree of biocompatibility – will probably contribute to increased AGE-formation (24). On the other hand, new technologies concerning certain high flux membranes, vitamin E-coated low-flux dialyzers and convective therapies may lead to less oxidative stress and enhanced AGE removal in hemodialysis patients (25). Preliminary evidence suggests that high-flux hemodialysis, and the use of low glucose dialysates in peritoneal dialysis are associated with lower levels of skin AF (Arsov Z et al. and McIntyre N et al, unpublished data).

Renal transplantation results in a decrease in AGE accumulation, though AGE levels remain well above those of controls. Moreover, the degree of AGE accumulation could be involved in the development of cardiovascular disease and chronic renal

transplant dysfunction after renal transplantation (26,27). Increased levels of skin AF are associated with several risk factors for chronic renal transplant dysfunction and cardiovascular disease (28). Unpublished data show an independent predictive value of skin AF for the development of chronic transplant dysfunction, which converge with the pathophysiological mechanism of oxidative stress and AGE accumulation in the outcome of graft loss in renal transplant recipients (29). Therefore, it can be hypothesized that preventive therapy with AGE inhibitors might be helpful in preserving renal function in these transplant patients. Again it should be stressed, that this hypothesis needs confirmation, and the relative importance of these risk factors weighed against the impact of other risk factors.

Skin AF and renal function in a screening setting: are skin AF levels directly related to the degree of renal failure?

Indeed, skin AF is correlated to the estimated GFR (eGFR) category, calculated with the Modification of Diet in Renal Disease (MDRD) – formula (using the re-expressed four-variable MDRD) (30), when performing global tests, as shown in the example presented below (Table 1; hitherto, unpublished data). The MDRD was used as a screening instrument in a large cohort of subjects with type 2 diabetes mellitus (n = 973), participating in the ZODIAC trial.

Table 1. skin AF and MDRD**.** Compare means of skin AF by One Way Anova: MDRD category 1 versus 2: p=0.39; 1 versus 3 and 4: p<0.001; 2 versus 3 and 4: p<0.001; 3 versus 4: p=0.009.

MDRD category (ml/min/1.73m^2)	N	Skin AF (mean±SD) (Arbitrary Units)	Age (mean±SD) (years)
1. eGFR ≥ 90	38	2.41±0.66	56±11
2. 60 < eGFR ≤ 90	578	2.64±0.70	63±11
3. 30 < eGFR ≤ 60	351	3.05±0.81	73±9
4. 15 < eGFR ≤ 30	6	4.02±1.07	76±6

At first sight, such results appear to vindicate the supposed correlation between eGFR and skin AF. However, one has to keep in mind in this assessment that the MDRD formula has not been sufficiently validated as a screening tool in subjects older than 70 years old (43% of our study population). Secondly, one should bear in mind, that age plays an important role in the MDRD-formula, and that age in itself is one of

the factors related to AGE accumulation. For statistical reasons, the eGFR cannot be corrected for age to allow a more reliable assessment. Therefore, we divided the same cohort into five groups according to age, and dichotomized the results under and above the median of eGFR (Table 2).

Table 2. Skin AF and eGFR in dichotomized age groups (I = mean skin AF in patients below the median of the eGFR; II = mean skin AF in patients above the median of the eGFR).

Age category (years)	N	Median eGFR (ml/min/1.73)	I Mean skin AF ± SD (Arbitrary units)	II Mean skin AF ± SD (Arbitrary units)	p-value *
< 50	81	76.2	2.08±0.51	2.28±0.48	0.075
50 ≤ age < 60	199	72.1	2.69±0.70	2.46±0.71	0.024
60 ≤ age < 70	278	65.7	2.73±0.74	2.64±0.74	0.282
70 ≤ age < 80	307	58.3	3.21±0.81	2.87±0.66	<0.001
≥ 80	108	55.9	3.25±0.85	3.10±0.57	0.305

SD, standard deviation; * Compare means of skin AF by One Way Anova.

Figure 2 shows the correlation between skin AF and MDRD for the entire patient group. As can be seen, the dichotomized results do not show a definite and consistent correlation between AFR readings and eGFR. Furthermore, Figure 2 speaks for itself when assessing the relationship between skin AF and eGFR in a large cohort. The r-square value of 0.106 ($p<0.001$) is rather disappointing. These results suggest, that skin AF is not a factor which is strongly associated with renal function, at least not in a screening setting in patients with type 2 diabetes.

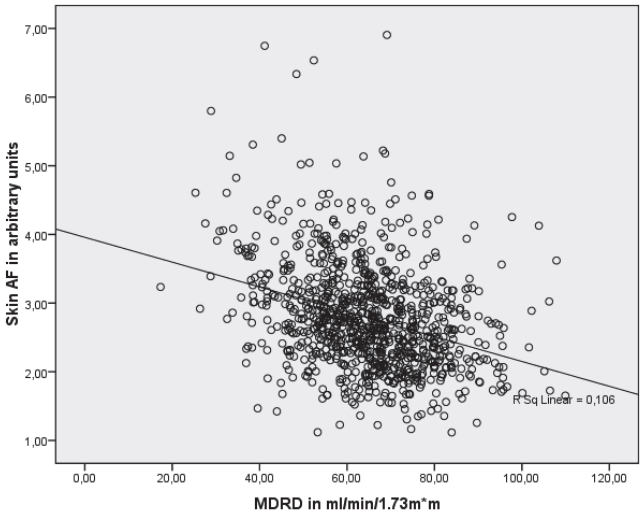

Figure 2. Scatterplot MDRD versus Skin AF

Conclusion

Skin autofluorescence is a new measurement that may have prognostic utility. This noninvasive and non-time-consuming method has been studied mainly in end stage renal disease and diabetes mellitus, but studies are ongoing in other patient groups as well. Based on the published (but still partly incomplete) evidence, skin AF, as assessed in different disease states: diabetes mellitus, renal failure, rheumatoid arthritis, is related to and predictive of morbidity and mortality.

The significance of skin AF as a meaningful tool in screening whole populations remains to be defined yet. More research is still needed in other patient populations in order to further delineate the exact role of both tissue AGEs and skin AF under various conditions. More transversal and longitudinal studies need to be done before the AFR can be used as a risk assessment tool in individual patient care. However, the potential exists. Maybe in the future, when drugs reducing AGE formation, AGE breakers, or dialysis modalities reducing AGE formation will become part of the therapeutic inventory, skin AF could offer a tool to identify responders to therapy and to monitor treatment as well.

Conflicts of interest

A.J. Smit is one of the founders of DiagnOptics B.V., The Netherlands, manufacturer of the AGE-Reader, which is based on the prototype used in the present article.

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CHAPTER 7

SKIN AUTOFLUORESCENCE AS A MEASURE OF ADVANCED GLYCATION ENDPRODUCT DEPOSITION: A NOVEL RISK MARKER IN CHRONIC KIDNEY DISEASE

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Curr Opin Nephrol Hypertens 2010, 19: 527 – 533

Abstract

Purpose of review Skin autofluorescence (SAF) is a new method to noninvasively assess accumulation of advanced glycation end products (AGE) in tissue with low turnover. Recent progress in the clinical application of SAF as a risk marker for diabetic nephropathy as well as cardiovascular disease in nondiabetic end-stage kidney disease, less advanced chronic kidney disease, and renal transplant recipients is reviewed.

Recent findings Experimental studies highlight the fundamental role of the interaction of AGEs with the receptor for AGEs (RAGEs), also called the AGE-RAGE axis, in the pathogenesis of vascular and chronic kidney disease. SAF predicts (cardiovascular) mortality in renal failure, and also chronic renal tranplant dysfunction. Long-term follow-up results from the Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) suggest that AGE accumulation is a key carrier of metabolic memory and oxidative stress. Short-term intervention studies in diabetic nephropathy with thiamine, benfotiamine and angiotensin-receptor blockers aimed at reducing AGE formation have reported mixed results.

Summary SAF is a noninvasive marker of AGE accumulation in tissue with low turnover, and thereby of metabolic memory and oxidative stress. SAF independently predicts cardiovascular and renal risk in diabetes, as well as in chronic kidney disease. Further long-term studies are required to assess the potential benefits of interventions to reduce AGE accumulation.

Introduction

The measurement of skin autofluorescence (SAF) has become a noninvasive method of assessing the accumulation of advanced glycation endproducts (AGEs) as a marker of the long-term impact of glycemic and oxidative stress in humans. Interest in AGEs as a central marker of the so-called metabolic legacy effect has expanded in the context of assessing the long-term effects of early intensive glycemic control in diabetes as well as the metabolic effects of chronic kidney disease (CKD) and chronic renal transplant dysfunction (1,2*). Kern et al. (3*) recently reported data supporting earlier observations identifying the predictive value of AGEs and AGE fluorescence in diabetic kidney disease in the DCCT-EDIC study (4). The AGE-RAGE axis is important in many forms of renal disease and suggests new approaches for intervention (5**,6*).

Advanced glycation endproducts: formation and effector pathways

AGEs are formed slowly by the Maillard reaction which is dependent on glucose levels, but rapid formation of AGEs occurs via another pathway involving reactive carbonyl compounds (RCCs) such as (methyl)glyoxal (the so-called dicarbonyl stress) during oxidative stress. The glyoxalase system forms a defense mechanism against this pathway (7). A third source of AGEs in humans is the intake of exogenous AGEs from food and smoking (8). Overall, accumulation of AGEs on proteins with low turnover may result from all these three sources: slow glycation, rapid formation via RCCs, and exogenously derived AGEs. When proteins are degraded to the so-called glycation-free adducts and glycation adduct residues, the former in particular are subsequently excreted via the kidney. In the presence of renal failure, this excretion mechanism fails or is overloaded, and further accumulation on proteins with low turnover may occur. Dicarbonyl stress is also increased in renal failure (9*).

AGEs are no innocent bystanders, but exert effects via two pathways. Firstly, they crosslink proteins, nucleic acids and lipids, resulting in structural changes, malfunction, and reduced breakdown. Mitochondrial glycation may enhance

oxidative stress, thereby introducing a vicious circle. Secondly, AGEs link to cell membrane receptors, the best known of which is the receptor for AGEs (RAGEs). This may lead to activation of intracellular pathways [including prolonged activation of nuclear factor-kappa B (NF-κB)], and release of cytokines (5**,10*) which may induce endothelial dysfunction and other deleterious vascular effects (11).

Development and validation of skin autofluorescence as marker of tissue advanced glycation endproduct accumulation

Several groups have developed devices to easily and rapidly measure SAF for the assessment of AGE accumulation in a point of care setting. In 1997, Jager first noticed increased fluorescence of skin in diabetic patients during noninvasive capillary microscopy, especially in those with complications. This led to the development of the so-called autofluorescence reader, a prototype of the AGE reader (DiagnOptics Technologies, Groningen, The Netherlands), which became commercially available in 2006. Initial results in patients with diabetes were reported in 1999 and were followed in 2004 by several publications mainly in the field of diabetes and renal failure (12-15). The principle of the method used in the AGE reader is shown in Figure 1. (16,17).

Clinical use of a second device that also utilises SAF to assess AGEs, the so-called SCOUT device (VeraLight, Albuquerque, New Mexico, USA) was first reported in 2007 (18). The intention of the SCOUT device is to diagnose diabetes. Maynard compared SAF with HbA1c and fasting plasma glucose for the detection of diabetes (confirmed by glucose tolerance test) in a naive population-derived cohort and found superior performance with SAF. No applications in renal failure have been reported so far. Recently, Xu (19) reported the development of a SAF device, but no clinical data have been provided yet. These three devices, all measuring SAF, are proposed to assess accumulation of AGEs in skin tissue. In case of the AGE Reader, validation of the level of SAF against specific AGE molecules [pentosidine, carboxymethyllysine (CML) and carboxyethyllysine (CEL)] was reported in patients with diabetes, renal

failure and healthy controls (12,13). In a combined analysis, 76% of variance in SAF level could be explained by the variance in dermal pentosidine levels in skin biopsies. The molecular nature of collagen-linked fluorescence in diabetes and end-stage kidney disease (ESKD) has been partially characterized by the detection of a major fluorophore, LW-1, with a molecular weight of 623 Da (20).

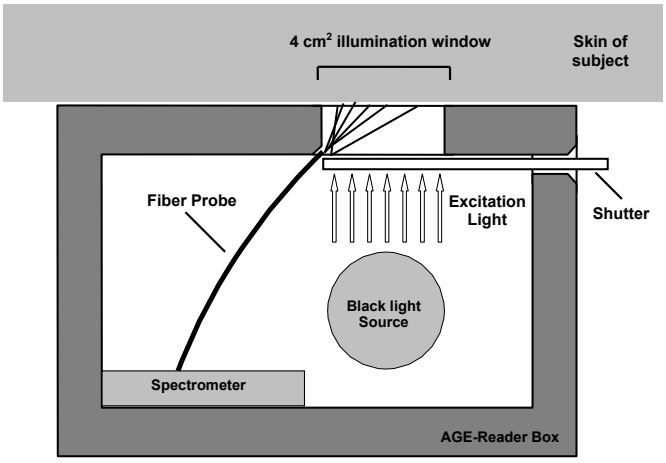

Figure 1a. Diagrammatic presentation of the AGE reader set-up. An ultraviolet-A light source with a peak wavelength of 370 nm illuminates a small skin area, the reflected and fluorescent light coming back from the skin is detected by a fiber and fed into a spectrometer for further analysis.

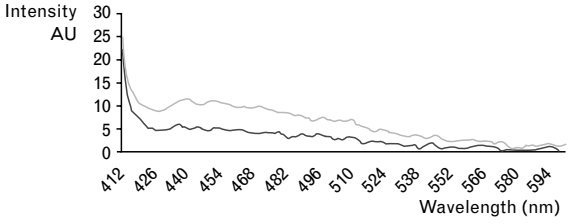

Figure 1b. Examples of the light intensity differences in skin fluorescence spectra in the 420-600 nm range between a patient with diabetes and a healthy control. The left side of the panel shows the peaked-off light intensities of the light source with a peak wavelength around 370 nm. Skin AF is the ratio between the light intensities in the 420-600 nm range divided by the light intensity in the 300-420 nm range.

AGEs are stable end products and form irreversible links in tissues with low turnover. The extent to which proteins are modified by accumulation of AGEs is an essential determinant of their functional and structural effects. Consequences of AGE modification are more important in tissue with low turnover, such as the dermis and glomerular and tubular basement membranes, than in blood or urine. Januszewski et al. (21) showed a strong correlation between SAF and AGE-related fluorescence of the eye lens, another tissue in which proteins with low turnover are present. Examples of how SAF may reflect functional and structural tissue damage better than plasma AGE levels are discussed below.

Advanced glycation end products and receptor for advanced glycation end products in renal disease

Diabetic nephropathy is the classical model for demonstrating the pathogenetic role of AGEs and RAGEs. Glomerulosclerosis in diabetic animals is associated with AGE deposition in mesangium as well as hyalinized and/or sclerotic lesions. Mesangial cell function is also altered after modification of collagen IV by glucose or methylglyoxal (22*). Overexpression of RAGEs accelerates the development of glomerulosclerosis in mice (23). In RAGE knockout models, and during inhibition of AGE formation, the development of diabetic nephropathy, with respect to both micro-albuminuria and renal function loss, is prevented (see further below) (24*).

AGEs also accumulate in nondiabetic uremic patients despite their normal serum glucose levels. Among hemodialysis patients, both diabetic patients and nondiabetic individuals have high plasma pentosidine and CML levels. Patients with less advanced stages of CKD show a relation between AGE levels (CML) and renal function in selected groups and in the general population (25). Hou et al. (26) proposed that AGEs and RAGEs may contribute to amplification of inflammation in nondiabetic CKD. Plasma pentosidine levels and RAGE expression on monocytes strongly increased in parallel with tumor necrosis factor, neopterin and CRP levels in patients with worsening (nondiabetic) CKD.

In diabetic and nondiabetic patients with ESKD, peritoneal dialysis treatment causes low-grade chemical peritonitis due to the limited biocompatibility of peritoneal dialysis fluids that contain high glucose concentrations and, therefore, glucose-derived products that are precursors of AGEs. Because RAGE is expressed on endothelial and mesothelial cells, the receptor may bind AGEs generated endogenously or formed during peritoneal dialysis. The binding of AGEs to RAGEs produces a local inflammatory reaction, likely as a consequence of vascular cell adhesion molecule-1 overexpression, leukocyte adhesion, and cytokine release.

The role of RAGEs in the pathogenesis of CKD has been reviewed in detail recently (5**) and in the broader context of vascular disease by Yan (10*) from the same group. A more critical view on the relevance of RAGEs in the development of ESKD has been provided by Thornalley and Rabbani (9*), who question the relevance of the experimental studies with often highly glycated AGE-modified proteins for development of human renal failure when plasma protein glycation is less marked. However, it must be noted that the degree of AGE modification of proteins in tissues with low turnover may be considerably higher than in plasma. Thornalley and Rabbani draw attention to another potentially relevant function of the RAGE receptor: decreased expression of glyoxalase 1, a component of antiglycation defense mechanisms in response to S100A12 protein, making the vasculature vulnerable to dicarbonyl stress and related AGE formation.

Skin autofluorescence in diabetic nephropathy, and prediction of macrovascular complications

Initial clinical studies were performed in diabetes mellitus, because it is the classic example of increased formation and accumulation of AGEs. SAF was indeed found to be approximately 30% higher in patients with type 1 or type 2 diabetes mellitus compared to age-matched controls. In a large cohort of well controlled primary care type 2 diabetes patients, the presence and degree of microvascular and macrovascular complications were associated with a graded increase in SAF (15). Gerrits et al. (27)

reported that SAF is not only associated with diabetic nephropathy, but that it is also a predictor of its development.

SAF was also an independent and strong predictor of macrovascular complications as well as mortality. Compared to the different variables in the UKPDS risk engine, SAF was the best single predictor, after calendar age, of total and cardiovascular mortality. SAF also adds predictive value to the UKPDS risk engine, resulting in risk reclassification in 25 – 30% of patients (28**).

The association of SAF with diabetic nephropathy was recently extended to type 1 diabetes (29). Additionally, Conway et al. (30) showed a strong correlation of SAF, as measured with the SCOUT device, with coronary artery calcification in type 1 diabetes (with a high discriminative ability of SAF to detect CAC scores > 400 (area under the curve of receiver operating characteristic curve 85%).

Skin autofluorescence in renal disease

High levels of SAF, even substantially higher than in patients with diabetes and complications, have been reported in several hemodialysis cohorts (13,31,32). Diabetic hemodialysis patients had slightly higher levels than nondiabetic hemodialysis patients. McIntyre and Chesterton (33) extended these observations to peritoneal dialysis patients and reported similarly increased SAF values. In peritoneal dialysis patients, SAF correlated with dialysis vintage and also with previous peritoneal dialysis glucose exposure, whereas there was no difference between diabetic and nondiabetic peritoneal dialysis patients. During a follow-up period of three years, SAF appeared to be a strong predictor of mortality in hemodialysis patients, independent of previous cardiovascular disease (13). In a report from the Renal Risk in Derby (RRID) primary care cohort, McIntyre reported that SAF was related to estimated glomerular filtration rate (eGFR) in CKD stage 3, independent of age, the presence of diabetes and smoking [McIntyre, Determinants of skin autofluorescence in CKD stage 3 patients, Poster F-PO1124, American Society of Nephrology, 2009]. SAF was

also independently associated with a history of cardiovascular diasease. In another cohort of type 2 diabetes patients and moderate nephropathy, Gerrits et al. (32) found that SAF was correlated with eGFR categories. Chabroux (29) confirmed the association of SAF with diabetic nephropathy in type 1 diabetes patients.

In hemodialysis patients, SAF is also related to several markers of cardiovascular dysfunction. SAF was independently associated with diastolic left ventricular function, whereas plasma AGEs were not (34). Ueno et al. (31) found that SAF is strongly and independently associated with pulse wave velocity (PWV) as a marker of arterial stiffness in ESKD (but also in controls). Ueno et al. also recently reported that in patients with ESKD, both SAF and serum pentosidine correlated with carotid intima media thickness (IMT), and that SAF was inversely related to endothelial progenitor cell (EPC) levels, whereas such a correlation was not observed with serum pentosidine. In multiple regression analysis SAF, but not serum pentosidine or IMT, was related to EPC levels (35*). In the RRID cohort, McIntyre found a positive and independent correlation between SAF and PWV [McIntyre, Determinants of skin autofluorescence in CKD stage 3 patients, Poster F-PO1124, American Society of Nephrology, 2009]. Thus, in patients with CKD, SAF allows the identification of patients with the most marked cardiovascular dysfunction.

In hemodialysis patients, a population with a high annual mortality, SAF is an independent indicator of those at highest risk. However, the clinical value of this information may seem limited in the absence of effective treatments. Conventional methods of renal replacement therapy are only partially effective with regard to AGE clearance, and the degree of removal is dependent on the frequency as well as the duration of dialysis (36). Furthermore, the dialysis procedure itself may contribute to AGE accumulation: oxidative stress is an important factor leading to AGE formation, and hemodialysis membranes, depending on their degree of biocompatibility, probably contribute to increased AGE formation as well. On the other hand, new technologies including high-flux membranes, vitamin E-coated low-flux dialyzers and convective therapies may provoke less oxidative stress and result in enhanced AGE removal in hemodialysis patients (37). Preliminary evidence suggests that high-flux hemodialysis and the use of low-glucose dialysate in peritoneal dialysis are associated with lower levels of circulating AGEs and SAF [Arsov S, manuscript in preparation].

Peritoneal dialysis solutions with a lower glucose content may also reduce serum AGEs as a result of reduced glycemic stress. The use of peritoneal dialysis fluids low in glucose degradation products results in prolonged technique survival and (more importantly) also significant patient survival (38). Preliminary data show that SAF is also lower in peritoneal dialysis patients on low or no glucose- containing dialysate (McIntyre N et al., unpublished data). These findings suggest that lower tissue AGE accumulation over time could be due to lower serum AGE levels and may reduce tissue damage.
The results of ongoing studies, investigating the independent predictive value of SAF for cardiovascular risk and further renal function loss in earlier stages of CKD, are awaited to assess its clinical value.

Our study group has also investigated the reversibility of AGE accumulation in renal transplantation. Kidney transplantation represents one of the most effective approaches to reduce the markedly increased AGE accumulation in dialysis patients, although AGE and SAF levels remain well above those of controls. Moreover, the degree of persisting AGE accumulation after renal transplantation might be involved in the accelerated development of cardiovascular disease and chronic renal transplant dysfunction (39). Increased levels of skin AF are indeed associated with several risk factors for chronic renal transplant dysfunction and cardiovascular disease (40). Importantly, in a large group of patients with a previous kidney transplantation, SAF was found to be a strong predictor of chronic transplant dysfunction and mortality in the following five years (2*). This strongly suggests that AGEs play an important role in the development of chronic transplant dysfunction and mortality, probably by accelerating systemic and renal atherosclerosis. SAF may prove valuable for assessing this risk in the post-transplant period.

Interventions to reduce advanced glycation end products and skin autofluorescence levels as well as clinical end points in renal disease.

Several approaches directed at reducing the effects of AGEs have been evaluated in models of diabetic nephropathy for reduction of microalbuminuria or slowing

down renal function decline. In experimental studies by Tan et al. (24*) in an obese diabetic mouse model, suppression of RAGE expression within a RAGE -/- genotype, and adminstration of alagebrium, an inhibitor of AGE accumulation, both and additively prevented renal damage, whereas feeding a low-AGE diet did not. The classic anti-AGE compound, aminoguanidine, an inhibitor of AGE formation, reduced microalbuminuria in experimental models, but has not been succesful in clinical trials because of side-effects (41). Novel AGE breakers, such as TRC418, have shown reduction of albuminuria and renal function loss in diabetic rats and are in development in human studies (42). In a merged dataset from two clinical studies, pyridoxamine was reported to reduce change from baseline in serum creatinine, but not microalbuminuria (43). Further development was, however, halted due to side-effects. Furthermore, aggravation of renal damage was found in other animal models when pyridoxamine was used in combination with ACE inhibitors. Thornalley and coworkers (44) reported a protective effect of thiamine and benfotiamine in a rat model of diabetic kidney disease. They also reported a modest reduction of microalbuminuria in a small short-term study in type 2 diabetes patients using high-dose thiamine (45), but Alkhalaf et al. (46) failed to find reduction of microalbuminuria or urinary excretion of the tubular damage marker KIM-1 in type 2 diabetes patients. Experimental studies using ACE inhibitors or angiotensin-receptor blockers (ARBs) to inhibit the renin-angiotensin-aldosterone system (RAAS) have reported reduced AGE formation (47). This effect of RAAS inhibition is at least partially modulated by the RAGE receptor. Several experimental studies using ARB treatment have shown reduction in tubular and glomerular AGE accumulation along with reduction of renal function loss, tubular damage parameters and proteinuria (48). In one small (n=11 diabetic retinopathy patients) 12-week clinical study, candesartan reduced urinary CML excretion, but not albuminuria. No other formal controlled studies of ARB treatment and its effect on AGE formation in diabetic nephropathy have been performed, but in a recent post-hoc analysis of the Irbesartan Diabetic Nephropathy Trial study in patients with type 2 diabetes and nephropathy, irbesartan did not alter the increase in pentosidine and CML in serum and gave only minimal reduction in renal function loss after 2 years of treatment (49).

One of the fundamental problems with these intervention studies may be their very short time frame: it seems improbable that a 3-month or even a 2-year treatment period would result in improvement in a condition associated with the accumulation of AGEs in tissues with low turnover, such as the basement membrane of the glomeruli or tubules, that may have taken years to provoke microalbuminuria or renal function loss. There is an urgent need for long-term studies (>2 years) which are aimed at preventing microalbuminuria and renal function loss rather than reversing it. In nondiabetic CKD the role of low-AGE diets should also be explored in long-term studies. Dietary AGEs seem to exert negative effects, especially once urinary excretion of AGE-free adducts becomes reduced with loss of glomerular filtration capacity (50). Finally, the predictive role of SAF levels for mortality and chronic graft dysfunction several years after renal transplantation, supports the concept of limited and slow reversibility of AGE-induced damage.

Conclusion

AGEs play a pivotal role in the development and progression of diabetic nephropathy, but also of nondiabetic CKD. SAF has been validated as a simple, noninvasive method for assessment of AGE content in tissue with low turnover. SAF as marker of AGE accumulation is a strong and independent predictor of nephropathy and also cardiovascular complications in diabetes. Similarly, in nondiabetic CKD, SAF is related to vascular dysfunction and predicts mortality in ESKD. After renal transplantation, SAF is a valuable predictor of chronic transplant dysfunction and mortality. The investigation of interventions aimed at reducing AGE accumulation in patients with renal damage should move from short-term studies in patients with established renal damage to long-term prevention in an early phase of diabetes or CKD.

Disclosure

A.J. Smit is founder and shareholder of DiagnOptics Technologies BV (Groniingen, The Netherlands), which developed the AGE reader.

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CHAPTER 8

INCREASED SKIN AUTOFLUORESCENCE: A PRONOUNCED MARKER OF MORTALITY IN HEMODIALYSIS PATIENTS

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Abstract

Background Accelerated formation and tissue accumulation of advanced glycation endproducts (AGEs), reflecting cumulative glycemic and oxidative stress, occurs in age-related and chronic diseases like diabetes mellitus (DM) and renal failure, and contribute to vascular damage. Skin autofluorescence (AF), a non-invasive measurement method, reflects tissue accumulation of AGEs. The aim of our study was to determine the predictive value of skin AF on overall and cardiovascular mortality in hemodialysis patients.

Methods Baseline skin AF was measured in 105 patients on hemodialysis, 23 had DM. Survival status was assessed after a median follow-up period of 4.9 years (interquartile range: 2.3 – 6.9 years).

Results Multivariate Cox regression analysis showed skin AF [hazard ratio (HR) 1.83; 95% confidence interval (CI) 1.32 – 2.54], pre-existing cardiovascular disease [HR 2.77; 95% CI 1.48 – 5.18], renal replacement therapy duration [HR 1.10; 95% CI 1.01 – 1.19], age [HR 1.03; 95% CI 1.01 – 1.06], serum albumin [HR 0.90; 95% CI 0.85 – 0.95], hematocrit [HR 0.92; 95% CI 0.86 – 0.98], phosphorus [HR 2.01; 95% CI 1.15 – 3.49], and parathyroid hormone (PTH) [HR 0.99; 95% CI 0.98 – 0.996] to be predictors of mortality, whereas DM was not. Pre-existing CVD and serum phosphorus were the only predictors of cardiovascular mortality.

Conclusion Skin AF showed to be an independent predictor of overall mortality in hemodialysis patients, but it had no predictive value for cardiovascular mortality.

Introduction

Overall and cardiovascular mortality rate is much higher in subjects with end stage renal disease compared to the general population (1-4). A contributing factor to the development of vascular damage is the formation and accumulation of advanced glycation endproducts (AGEs), which progressively occurs in all individuals with ageing. Accelerated formation and tissue accumulation of AGEs on proteins with slow turnover, occur in patients with chronic diseases like diabetes mellitus (DM), chronic renal failure, neurodegenerative diseases, and systemic inflammatory diseases. These AGEs are formed non-enzymatically under circumstances of glycemic or oxidative stress. Compared to healthy subjects, increased oxidative stress and reduced antioxidant levels have been found in patients with chronic kidney disease (CKD) or those on hemodialysis. Oxidative stress, which accompanies uremia, increases the inflammatory state and promotes the alterations of certain molecules such as proteins, lipids, and carbohydrates (5-7). Additionally, the impaired excretion of the breakdown products of AGE cross-linked proteins, the so-called AGE free peptides and adducts, further contributes to the accumulation of AGEs in patients with chronic kidney disease. AGEs formed under pro-inflammatory and pro-oxidative circumstances will contribute to endothelial dysfunction, and are associated with the occurrence of cardiovascular disease (CVD) (8,9).

A substudy of the Diabetes Control and Complications Trial (DCCT) showed that AGE levels in skin biopsies predicted the risk of development or progression of microvascular disease in type 1 DM, an observation which is suggestive for the deleterious effects of AGEs on the vascular wall (10).

Tissue accumulation of AGEs can be assessed in skin biopsies, but this is an invasive method. The autofluorescence (AF) reader, a noninvasive device, is based on the fluorescence properties of certain AGEs. The degree of skin AF reflects the level of tissue accumulation of AGEs reasonably well. This method has been validated against specific AGE levels in skin biopsies in patients with diabetes, patients on hemodialysis and in healthy control subjects (11,12). Cross-sectional and longitudinal analyses have already shown the relationship between skin AF as a marker of tissue AGEs, and vascular morbidity and mortality in DM, and mortality in hemodialysis. Cross-sectional

analyses also showed the association of skin AF with renal and cardiovascular risk factors in stage 3 and pre-dialysis CKD patients (12-18).
The aim of the present study was to address the predictive value of skin AF on overall and cardiovascular mortality in hemodialysis patients.

Subjects and Methods

Study Group
Between August 2003 and February 2004, 105 hemodialysis patients who dialyzed in the hemodialysis centre of the Isala Clinics in Zwolle, The Netherlands, were included in the study cohort. These hemodialysis patients had a three times weekly scheme with variable duration of dialysis, all were using biocompatible low-flux membranes. Patients with Fitzpatrick class V-VI skin type were excluded from participation, because of the reduced ability of the prototype autofluorescence reader to reliably measure autofluorescence in these dark skin types. This inability is due to the high absorption of both the excitation and emission light when using an ultraviolet light source with a certain peak intensity (19,20).
At the end of follow-up, January 2011, survival status of all patients was assessed. Approval by the local ethic committee had been obtained and informed consent was given by all of the included patients.

Data collection and definitions
Clinical data and laboratory results were obtained at the time of the baseline skin AF measurement. Laboratory data included serum non-fasting total cholesterol, creatinine, urea, albumin, hemoglobin, hematocrit, calcium, phosphorus, parathyroid hormone, all measured according to the standard laboratory procedures. Physical assessment data included body mass index (BMI) and blood pressure. Blood pressure assessment was the average of three measurements obtained after disconnection of the hemodialysis session in supine position, using an aneroid device, in the week before the skin AF measurement.

Diagnosis of DM was confirmed using ADA criteria. The European Dialysis and Transplant Association Codes were used to define the primary diagnosis of renal failure, subdivided in divisions: primary glomerulonephritis, interstitial nephropathies, multisystem diseases, DM and not known/other. Some DM patients needed dialysis due to another cause of renal disease than diabetic nephropathy.
CVD was defined as: Ischemic heart disease (International Classification of diseases (ICD-9) codes 410 – 414 and/or a history of coronary artery bypass surgery or percutaneous coronary intervention), cerebrovascular accidents or peripheral vascular disease (clinical history of intermittent claudication, percutaneous transluminal angioplasty, bypass surgery and/or limb amputation). The presence of macrovascular complications was assigned when meeting at least one of the criteria for CVD.

Skin autofluorescence
Skin AF was measured at the lower, non-fistula arm by the AF reader (prototype of the current AGE Reader; DiagnOptics BV, Groningen, The Netherlands), a noninvasive device which illuminates a skin surface of ~ 4 cm^2, with an excitation ultraviolet light source with peak intensity at ~370 nm. Emission light and reflected excitation light from the skin are measured with a spectrometer in the 300 – 600 nm range. AF was computed by dividing the average light intensity of the emission spectrum (420 – 600 nm) by the average light intensity of the excitation spectrum (300 – 420 nm), multiplied by hundred and expressed in arbitrary units (AU). Assessing skin AF is not a time-consuming method and the operation of the device requires no special training or skills. Reproducibility has been tested before and the interindividual measurements and intraindividual seasonal variance showed a mean relative error of ~ 5% (21).

Statistical analyses
Student's t-test was used to compare groups with respect to quantitative variables, and Cox-regression analysis was used to estimate the effect and the 95% confidence interval (CI) of each predictor: skin AF, serum albumin, DM, pre-existing CVD, renal replacement therapy duration at baseline, age, pulse pressure, hematocrit, serum

phosphorus and parathyroid hormone (PTH) on overall and cardiovascular mortality, both in a univariate and in a multivariate model correcting for all other predictors.

Results

Baseline characteristics are shown in Table 1; patients were subdivided in a surviving patient group and a nonsurviving patient group. Mean age of all the 105 included patients was 65 years, 68% were male, and 93% were Caucasian (7% Asian). Causes of end stage renal disease were classified in 9% of all patients as DM, in 31% as hypertension or renovascular disease, in 21% as primary glomerular disorders and in 39% as having another primary disorder causing end-stage renal disease. There were 23 type 2 DM patients (there were no type 1 DM patients in our hemodialysis group at that time point) who appeared well controlled with a mean HbA1c ± SD of 6.8 ± 1.2% (51 ± 13 mmol/mol). Obviously, at baseline, the non-surviving patients were older, had a higher percentage of DM and pre-existing CVD, had a higher pulse pressure, a lower albumin level and a higher skin AF level. After a median follow-up time of 4.9 years (interquartile range: 2.3 – 6.9 years), the overall mortality rate was 66%, and cardiovascular mortality rate was 32%. Crude mortality rate was higher amongst the DM patients: 87% (20/23) versus 60% (49/82) in the non-DM group ($p = 0.02$).

Table 1. Baseline characteristics of hemodialysis patients (total, surviving patients and nonsurviving patients).

Characteristic	Surviving hemodialysis patients (n = 36)	Non-surviving hemodialysis patients (n = 69)	Total (n = 105)
Age (year)	54.5 ± 15.1	70.5 ± 10.9[a]	65.1 ± 14.6
Gender M/F (%)	58/42	73/27	68/32
Smoking (%)	8	17	14
BMI (kg/m^2)	25.5 ± 4.5	24.6 ± 4.5	24.9 ± 4.5
Systolic bloodpressure (mmHg)	140 ± 22	149 ± 25	146 ± 24
Pulse pressure (mmHg)	56 ± 18	70 ± 20[a]	65 ± 20
Median duration of renal replacement therapy in years (interquartile range)	2.94 (0.61 – 5.46)	2.56 (1.21 – 5.72)	2.64 (1.11 – 5.54)
Pre-existing CVD (%)	17	67[a]	50
Coronary heart disease (%)	8	45[a]	32
CVA/TIA (%)	8	20	16
Peripheral vascular disease (%)	3	30[a]	21
Diabetes mellitus (%)	8	29[b]	22
Creatinine (µmol/l)	983 ± 208	816 ± 232[a]	873 ± 237
Urea (mmol/l)	27.6 ± 5.4	26.0 ± 6.9	26.5 ± 6.4
Albumin (g/l)	40 ± 4	37 ± 5[c]	38 ± 5
Total Cholesterol (mmol/l)	3.9 ± 0.9	3.8 ± 1.0	3.8 ± 0.9
Hemoglobin (mmol/L)	7.9 ± 0.7	7.7 ± 0.9	7.8 ± 0.8
Hematocrit (%)	38.2 ± 3.8	37.8 ± 4.6	38.0 ± 4.3
Calcium (mmol/L)	2.36 ± 0.20	2.34 ± 0.20	2.35 ± 0.20
Phosphorus (mmol/L)	1.68 ± 0.43	1.63 ± 0.58	1.65 ± 0.53
Median parathyroid hormone (pmol/L)	19.7 (4.7 – 60.8)	15.6 (6.9 – 33.6)	16.9 (6.6 – 39.5)
Skin AF (AU)	2.74 ± 0.77	3.37 ± 0.86[a]	3.16 ± 0.88

Data are mean ± SD, unless otherwise indicated. Abbreviations: BMI, body mass index; CVD, cardiovascular disease; CVA/TIA, cerebrovascular accident/transient ischemic attack; AF, autofluorescence. [a]$p \leq 0.001$; [b]$p = 0.02$; [c]$p = 0.03$ (compared to the surviving hemodialysis patients)

Univariate Cox regression analysis showed that pre-existing CVD and skin AF were the most predictive markers of overall mortality: HR 3.44 (95% CI 2.07 – 5.74), and 1.72 (95% CI 1.32 – 2.23). DM status had statistically significant predictive value for overall mortality in univariate analysis, but not in multivariate Cox regression analysis (Table 2), whereas skin AF, serum albumin, pre-existing CVD, renal replacement duration at baseline, age, hematocrit, serum phosphorus and PTH showed significant predictive value for overall mortality in the multivariate analysis. Surprisingly, median (interquartile range) PTH levels of the survivors were higher than those of the nonsurvivors: 19.7 (4.7 – 60.8) pmol/L versus 15.6 (6.9 – 33.6) pmol/L.

Table 2. Predictors of overall mortality by univariate and multivariate Cox-regression analysis.

	Univariate		Multivariate	
Variable	**Hazard Ratio (95% CI)**	***p***	**Hazard Ratio (95% CI)**	***p***
Skin autofluorescence (AU)	1.72 (1.32 – 2.23)	<0.001	1.83 (1.32 – 2.54)	<0.001
Diabetes mellitus (yes versus no)	1.93 (1.15 – 3.26)	0.01	1.07 (0.61 – 1.87)	0.8
Pre-existing CVD (yes versus no)	3.44 (2.07 – 5.74)	<0.001	2.77 (1.48 – 5.18)	0.001
RRT duration at baseline (years)	1.01 (0.95 – 1.08)	0.7	1.098 (1.01 – 1.19)	0.02
Age (years)	1.05 (1.03 – 1.07)	<0.001	1.03 (1.01 – 1.06)	0.02
Pulse pressure (mmHg)	1.02 (1.01 – 1.03)	<0.001	1.01 (0.996 – 1.02)	0.2
Albumin (g/L)	0.93 (0.89 – 0.97)	0.001	0.90 (0.85 – 0.95)	<0.001
Hematocrit (%)	0.97 (0.92 – 1.03)	0.4	0.92 (0.86 – 0.98)	0.008
Serum phosphorus (mmol/L)	0.85 (0.51 – 1.40)	0.5	2.01 (1.15 – 3.49)	0.01
Parathyroid hormone (pmol/L)	0.99 (0.99 – 1.00)	0.07	0.99 (0.98 – 0.996)	0.003

Abbreviations: AU, arbitrary units; CI, confidence interval; CVD, cardiovascular disease; RRT, renal replacement therapy.

None of the variables showed significant predictive value for cardiovascular mortality in univariate Cox regression analysis. In multivariate analysis, only pre-existing CVD and serum phosphorus showed significant predictive value for cardiovascular mortality: HR 5.38 (95% CI 1.31 – 22.19) and HR 3.59 (95% CI 1.32 – 9.74).

During follow-up, 28 patients received a kidney transplant of whom 4 patients (1 DM, 3 non-DM) had died at the end of follow-up. Performing Cox regression analyses in the population after exclusion of the transplanted hemodialysis patients did not change the initially identified predictive markers of overall mortality.

Discussion

This study showed that skin AF was significantly higher in the nonsurvivor group compared to the survivor group. Baseline skin AF was a predictor of overall mortality and showed independent predictive value regarding overall mortality besides the well-known risk factors serum albumin, pre-existing CVD, renal replacement therapy duration at baseline, age, hematocrit, and serum phosphorus, with only little change in hazard ratio compared to the univariate analysis. DM per se, usually an important predictor of mortality in hemodialysis, did not independently have predictive power regarding mortality in the multivariate analysis, probably due to the small group size or to the more pronounced role of the other conventional and nonconventional cardiovascular risk factors in hemodialysis patients in general. We confirmed the independent predictive value of skin AF for overall mortality in hemodialysis patients as previous reported, but now with a slightly longer follow up (12). An explanation for the predictive value of skin AF, or AGE fluorescence, could be the intermediate role of AGEs in the development of vascular complications in DM, renal failure and CVD. Besides decreased clearance of AGEs in patients with renal failure, AGE formation is accelerated throughout the years during dialysis, resulting in progressive AGEs cross-linked to long-lived proteins embedded in tissue which may contribute to endothelial dysfunction. Additionally, high oxidative stress levels in patients with end-stage renal disease prior to the start of renal replacement therapy could contribute to increased AGE levels as well (6,9,22). Recent data showed that high skin AF levels, as reflecting AGE accumulation, were found in moderate renal failure as well and were associated with renal and cardiovascular risk factors (17).

In contrast to our findings, previous studies on serum AGEs did not show predictive value concerning (cardiovascular) mortality or morbidity in chronic kidney disease

(23,24). This might be due to AGE accumulation in skin collagen and other long-lived proteins which are supposed to remain more stable than serum AGE levels due to a variable clearance of circulating serum AGEs in patients with chronic kidney disease. Circulating serum AGEs apparently do not properly mirror chronic tissue AGE accumulation and its ensuing vascular damage on the long term. A recent report observed a relationship between diastolic dysfunction and skin AF in hemodialysis patients, whereas serum AGEs did not support this concept (25). Another explanation for the higher variability of circulating serum AGEs in hemodialysis patients may be the use of different dialysis modalities (26-28).

Both PTH and phosphorus are well-known risk factors for mortality in hemodialysis patients. Although the two variables are most probably depending on each other, they both have their independent effect on mortality. PTH was negatively associated with mortality, with a wide spread of PTH levels in our study group, and with surprisingly higher levels of PTH in the survivor group. This paradoxical finding could be explained by the fact that part of the patients underwent a parathyroidectomy because of tertiary hyperparathyroidism, and lost their ability to produce large amounts of PTH, thus masking a possible correlation between severe parathyroid dysfunction and increased mortality risk.

Several factors may play an important role in the pathogenesis of the so-called 'chronic kidney disease'-associated wasting. Hypoalbuminemia is just one aspect occurring in correlation with those factors and therefore in line with our finding of being associated with mortality. Other factors contributing to protein-energy wasting in chronic kidney disease include systemic inflammation, changes in appetite-controlling hormones from reduced renal clearance, aberrant neuropeptide signalling, insulin and insulin-like growth factor resistance, and metabolic acidosis (29,30). Consequently, hemodialysis patients are exposed to chronic inflammation, which might contribute to or overlap with the deleterious effects of oxidative stress in the development of arterial vascular disease. Unfortunately, we did not have sufficient data regarding nonconventional cardiovascular markers of oxidative stress or inflammation (like hs-CRP and IL-6).

There are some technical restrictions of the skin AF measurement. The skin AF reader may also measure other fluorophores than fluorescent AGEs, and there

are nonfluorescent AGEs present in the skin as well that may contribute to the overall effects of AGE accumulation. Nevertheless, the predictive value of skin AF is repeatedly confirmed in several studies (12-16). Moreover, only patients with skin type Fitzpatrick class I-IV could be included in our study at that time, because of the limitation of the prototype AF reader to measure in dark skin types. This issue was only just solved according to a recent report that skin AF can be measured in dark skin types with a newly developed AF reader (31). Other limitations of the study are the small sample size and the possible selection bias of the hemodialysis patients with DM. Skin AF measurements were performed during hemodialysis, which theoretically could temporarily increase skin AF levels in association with higher oxidative stress levels induced by dialysis. However, no changes in skin AF before and after hemodialysis sessions could be observed, both in high- and low-flux dialysis settings (unpublished data: Koetsier M: Optical skin spectra reflect changes in tissue after hemodialysis).

In our opinion, our study provides further support for the concept of chronic inflammation and oxidative stress, both contributors to accelerated AGE formation and accumulation, as important non-conventional cardiovascular risk markers of mortality in a hemodialysis population. Measuring skin AF is an elegant and noninvasive method for the assessment of the amount of tissue AGEs, contributing to identify the mortality risk in hemodialysis patients.

To summarize, risk factors which activate chronic inflammation and/or oxidative stress, contribute to the deleterious effects on the vascular wall in patients on hemodialysis. When correcting for other known risk factors, skin AF proved to be a pronounced marker of overall mortality in hemodialysis patients, whereas DM, another well known risk factor, did not play a role in our study group.

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Disclosure Statement

A.J. Smit is founder of DiagnOptics B.V., Groningen, The Netherlands, manufacturer of the AGE-Reader, which is based on the prototype used in the present article.

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CHAPTER 9

SUMMARY, DISCUSSION, AND FUTURE PERSPECTIVES

Summary

Increased glycemic and oxidative stress play a pivotal role in the pathogenesis of vascular damage in high risk groups such as patients with diabetes mellitus (DM) or chronic kidney disease, culminating in cardiovascular events, endstage renal disease (ESRD) and mortality.

Chronic exposure to glycemic and oxidative stress translates in often long lasting and cumulative changes. Such effects are also described as metabolic memory and can be subdivided in short-term and long-term metabolic memory. Short-term metabolic, specifically glycemic memory is reflected by e.g. the degree of glycation of hemoglobin, measured as HbA1c subfraction representing the degree of glycemic control over approximately the last 6 – 8 weeks. Long-term metabolic memory can be reflected by the accumulation of advanced glycation endproducts (AGEs) in tissue components with slow turnover, such as collagen in the dermis of the skin which has a lifetime of about 15 years. The relationship between poor metabolic control and the development or progression of diabetic complications has been established in type 1 DM in the Diabetes Control and Complications Trial (DCCT) and in type 2 DM in the United Kingdom Prospective Diabetes Study (UKPDS) during the nineties (1,2). The importance of long-term metabolic memory is highlighted in the long-term follow-up of both landmark trials which also showed an increasing benefit of good glycemic control over prolonged time (>10 years), even when the good glycemic control was limited to the initial intervention period at the early stage of diabetes (3,4).

Patients with chronic renal insufficiency have decreased renal clearance capacity of serum AGEs and AGE-free adducts, contributing to the accumulation of AGEs. Higher levels of AGEs may enhance oxidative stress, introducing a vicious circle. A treatment option for end stage renal disease such as hemodialysis, may in itself also induce repetitive bouts of oxidative stress and may reduce levels of protective antioxidants. All these imbalances of oxidative stress status and increased levels of AGEs will contribute to vascular morbidity and mortality in the long term.

Increased levels of AGEs may represent a useful marker of enhanced glycemic and oxidative stress, and moreover long-term metabolic memory. We assessed skin autofluorescence (AF), a noninvasive measurement reflecting tissue AGE accumulation.

P**art I** of this thesis outlines four longitudinal studies performed in a large cohort of type 2 DM patients derived from primary care. The included patients were all participating in a shared-care project of the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC) study, which started in 1998 and is still ongoing. Baseline skin AF measurements were performed in a cohort of 973 patients in 2001 – 2002, and a second skin AF measurement was assessed randomly in 452 patients from the initial population of 973 patients who were still participating in the shared-care project in 2004 – 2005. Baseline serum peroxiredoxin 4 (Prx4) levels were assessed in 1689 patients originating from two cohorts of the ZODIAC study: one cohort started at the beginning in 1998 and the other in 2001.

Chapter 1 outlines the biochemical process responsible for the formation and removal of AGEs, oxidative stress and the deleterious effects of AGEs on the vascular wall. It also outlines the assessment of AGEs and the development of the AGE reader, a non-invasive device which assesses skin AF.

Chapter 2 provides information about skin AGE levels measured by skin AF. This study describes the predictive value of skin AF for the development of microvascular complications in a well-controlled type 2 DM population. Skin AF was superior to that of many other traditional risk predictors like diabetes duration and HbA1c. Besides the independent predictive value for the development of any microvascular complication, skin AF also proved to be an independent predictor of the development of neuropathy and (micro)albuminuria when assessed in the equation as separate complications. These findings were in agreement with the DCCT/EDIC (Epidemiology of Diabetes Interventions and Complications) substudy, which also showed the predictive value of skin AGE levels for the development or progression of microvascular complications in patients with type 1 DM, even after adjustment for HbA1c (1,5).

In **Chapter 3** we investigated whether HbA1c assessments could predict the change in skin AGEs during time in type 2 DM. Different integrated assessments of HbA1c were used to investigate to what extent they could predict skin AF, as reflecting cumulative glycemic stress and therefore tissue AGE accumulation. Hypothetically, the accumulation of AGEs in type 2 DM should be predicted by the course of HbA1c during a certain period (probably years) prior to the skin AF measurement.

The different integrated assessments of HbA1c which were used were: the variance of HbA1c, mean HbA1c, maximum HbA1c, and HbA1c at baseline. Adjustments were made for variables which could affect the rate of formation and accumulation of AGEs during years: baseline skin AF (reflecting baseline AGE accumulation), age, diabetes duration, creatinine, and smoking. The results of this study showed that changes in skin AF are poorly predicted by the degree of (relatively short-term) glycemic control as assessed by HbA1c in a type 2 diabetes group. It is hard to define which marker for metabolic control over a certain period is the best predictor of the development of chronic diabetic complications.

In **Chapter 4** we prospectively investigated Prx4, a free serum antioxidant, and its association with cardiovascular and all-cause mortality in 1161 type 2 DM patients, all participating in the earlier mentioned ZODIAC study. For the first time, this study showed evidence that Prx4 was independently associated with increased risk for both cardiovascular and all-cause mortality in type 2 DM. Therefore, although our findings need to be confirmed by others, Prx4 may have the potential to become a novel cardiovascular biomarker.

In addition, we were able to show a normal life expectancy of type 2 DM patients treated in primary care, when compared to the general population of a Western European country **(Chapter 5)**. However, also in this large subgroup of patients, a history of cardiovascular disease and albuminuria still remained predictive for a reduction of life expectancy. Major changes in treatment regimens over the past ten years, with more stringent goals for all aspects of diabetes care, should have contributed to a more favourable metabolic memory state and could therefore contribute to the improvement of life expectancy in type 2 DM. Data published in the last decade already showed a decline in (cardiovascular) mortality rates amongst diabetic patients (6-10).

Part II, among others, describes the accumulation of AGEs, as reflected by skin AF, in patients with chronic renal failure and patients on hemodialysis.

Chapter 6 is an editorial comment about AGEs, as assessed by the AF reader, and renal function. It outlines the decrease in clearance of serum AGEs and AGE free adducts in patients with renal failure, resulting in AGE accumulation (11-13). It also underlines the importance of increased oxidative stress levels in patients with chronic kidney

disease or patients on hemodialysis contributing to the formation and accumulation of AGEs (14,15). Original data are shown about the possible role of skin AF and renal function in a screening setting in 973 patients with type 2 DM participating in the ZODIAC study. In contrast to expectations, skin AF did not show a definite and consistent correlation with the estimated Glomerular Filtration Rate [calculated with the Modification of Diet in Renal Disease (MDRD) formula] in this patient group. To explain this, one has to take into account three aspects of the MDRD formula. First, the MDRD formula has not been sufficiently validated as a screening tool in subjects over 70 years old (43% of our study population); secondly, age plays an important role in the MDRD formula, and finally, age in itself is one of the factors related to AGE accumulation.

Chapter 7 is an overview of the clinical application of skin AF as a marker of diabetic nephropathy as well as cardiovascular disease in nondiabetic ESRD, less advanced chronic kidney disease, and renal transplant recipients. This chapter makes a strong point for the hypothesis that AGEs play a pivotal role in the development and progression of diabetic nephropathy as wel as in nondiabetic chronic kidney disease. It also points out the predictive value of skin AF, as a marker of the accumulation of AGEs, for the development of diabetic nephropathy, for cardiovascular complications in patients with type 2 DM and for mortality in ESRD (16-20). Furthermore, it describes a critical approach towards the novel interventions to reduce AGEs.

In **Chapter 8** we investigated and discussed the predictive value of skin AF on overall and cardiovascular mortality in hemodialysis patients. Risk factors which activate chronic inflammation or induce increased levels of oxidative stress, contribute to an accelerated formation and tissue accumulation of AGEs. The accumulation of AGEs, as reflected by skin AF, finally leads to deleterious effects on the vascular system in hemodialysis patients. Baseline skin AF was significantly higher in the non-survivor hemodialysis group compared to the survivor group. Besides the conventional risk factors for mortality in hemodialysis patients, skin AF showed independent predictive value regarding overall mortality.

To summarize, this thesis describes the determinants of cardiovascular risk in two high risk patient groups: type 2 DM and patients with chronic kidney disease or ESRD. In these high risk patient groups, we showed evidence for skin AF as a marker of morbidity and mortality, we studied the association of the potentially novel biomarker Prx4 and mortality and we drew attention to predictors of mortality in type 2 DM patients compared to the general population.

Discussion

Skin AF measurement is based on the specific fluorescence characteristics of AGEs, reflecting the accumulation of AGEs. Skin AF has been validated against specific AGEs in skin biopsies (considered as the gold standard) in patients with diabetes and patients on hemodialysis as well as in healthy control subjects (20,21).
The DCCT skin collagen ancillary study group clearly showed the association of an intensive treatment period of hyperglycemia, compared to conventional treatment, with lower levels of AGEs in skin collagen. These AGE levels in skin biopsies also predicted the risk of development or progression of microvascular disease in type 1 DM, even after adjustment for HbA1c (1,5). In one of our studies, we found skin AF to have predictive value for the development of the composite endpoint microvascular disease in type 2 DM, and in particular for neuropathy and microalbuminuria. There was no prognostic value of skin AF for retinopathy, when adjusted for other variables. The latter negative finding could be due to different reasons. Firstly, diabetes duration or the follow-up period to develop retinopathy could have been too short to develop retinopathy; maybe retinopathy needs more time to develop. Secondly, a small number of patients developed retinopathy compared to the other complications, and finally, the pathogenesis of retinopathy may be different compared to that of nephropathy and neuropathy (22). Lack of retinopathy progression was also seen in the Medalist group (351 type 1 DM patients who have been followed at the Joslin clinic), despite extremely long diabetes duration (over 50 years) (23). This study suggests that protective factors may play a role in the lack of retinopathy progression, which could also apply to our findings. First, there could be neutralization of the initiating toxic

effects of hyperglycemia by unknown mechanisms. Secondly, unknown combating mechanisms responsible for progression of complications and even facilitation of glycemic or metabolic memory could play a role. There also might be endogenous protective factors on metabolic memory with certain AGEs playing a specific role, which could act as a protective factor in contrast to the deleterious effects of AGEs what we have found until now. Hypothetically, the underlying protective mechanisms also might have something to do with overexpression or upregulation of certain antioxidant system components. Unfortunately, the Medalist study examined serum AGE concentrations, whereas we measured skin AF as a better reflection of tissue AGE accumulation.

At the moment, the easily applicable and non-invasive technique of skin AF still might be the best alternative method to determine the accumulation of tissue AGEs in human in a patient-friendly way compared to the gold standard of skin biopsies. However, there are some important aspects about this method to consider. First of all, not all of the AGEs will be detected by this technique. Nonfluorescent AGEs like Nε-carboxymethyllysine (CML) and pyralline will not be measured by the AGE reader. Secondly, tissue components that fluoresce in the same range of wavelength might act as confounding fluorophores (e.g. NAD(P)H) and thus contribute to a higher variation in skin AF level. Furthermore, hemoglobin and melanin are also capable of absorbing light in the 330 – 400 nm range, which makes them possible disturbing factors for the skin AF measurement. Finally, high oxidative stress levels, irrespective of glycemic status, could result in high concentrations of oxidized endproducts. Higher skin AF levels have also been found under circumstances of septicemia or acute myocardial infarction, both involving high levels of oxidative stress (24,25). Notwithstanding, skin AF has shown to be a predictor of microvascular complications, cardiovascular events and mortality in diabetes and hemodialysis patients (16,18-20).
Besides the biomarker skin AF, we also have studied another potential biomarker in the same type 2 DM population: Prx4. This study provides indirect evidence that higher levels of oxidative stress, as reflected by serum Prx4, an enzyme of the antioxidant defense system, increases the cardiovascular risk. Unfortunately, we did not have a control group to compare with, but higher levels of serum Prx4, as

reflecting oxidative stress, were associated with an increased risk of cardiovascular and all-cause mortality. In 2007, Mulder et al. already showed the association of skin AF with markers of oxidative stress: serum levels of sRAGE and neopterin in predominantly euglycemic patients (26). To strenghten our findings, future research to investigate the association of skin AF and Prx4 in our type 2 DM population would be an interesting objective of study.

A few more key questions about skin AF remained unanswered, which will be discussed in the following paragraph. The recurring question remains whether skin AF measurement truly assesses AGEs which are thought to be ultimately responsible for the deleterious effects on the vascular wall. If this question can be anwered by yes, skin AF could be used as a true surrogate marker of vascular damage. Besides the directly damaging effects of AGEs, skin AF could also reflect a certain degree of protein modification which additionally is responsible for vascular damage. During the last few years, more confirming evidence of the association between skin AF and vascular morbidity and mortality in cross-sectional as well as in longitudinal studies has been reported. That makes skin AF a more plausible marker of vascular damage, which in turn could make skin AF a biomarker for long-term metabolic stress.
A second issue is whether the excitation of fluorescence indeed represents a true estimate of skin AGE accumulation and the AGE accumulation in the walls of all arterial vessels larger than capillaries. Skin AF has been validated against skin biopsies which correlated well with certain AGEs. To elucidate the second question, autopsy studies with the assessment of AGEs in different sizes of arterial vessel walls have to be done.
One also has to pose the question to what extent skin AF measurement is representative for the degree of endothelial damage of the vascular wall c.q. endothelial dysfunction, by the accumulation of AGEs. Besides skin AF, all endothelial function tests are limited in their clinical predictive value, but one could defend them by emphasizing their prognostic significance in predicting vascular events. Endothelial dysfunction is associated with an increased risk for cardiovascular events. Skin AF has shown to be associated with vascular morbidity and mortality in high risk patients groups (e.g. diabetes mellitus, ESRD, hemodialysis) (16,18-20,27). Skin

AF is also elevated in patient groups with inflammatory diseases, such as systemic lupus erythematosus, Wegener's granulomatosis, and rheumatoid arthritis (28-30). Therefore, these patient groups can be defined as high risk patient groups for cardiovascular morbidity and mortality as well.

Despite all the relationships that were found between skin AF and morbidity and mortality, we can not derive valid and reliable conclusions for an individual patient level. Moreover, because of the considerable overlap in measurement outcomes between subjects with and without incidents during longitudinal follow up, one should keep a critical attitude towards the possible predictive value of skin AF in the individual patient. In our studies we did not investigate the additional value of skin AF in the prediction of mortality compared to the traditional cardiovascular risk factors such as systolic bloodpressure, cholesterol and albuminuria. Lutgers, meanwhile, studied the additional clinical value of skin AF in the evaluation of risk for fatal and non-fatal cardiovascular events and total mortality in type 2 DM. Reclassification of cardiovascular risk of type 2 DM patients with a UKPDS risk score < 10% and skin AF levels above the median resulted in higher cardiovascular risk ranking. This study was limited by a relatively short follow-up period and was too small compared to larger studies which are specifically designed to develop risk-prediction models (19). For the individual patient, the additional value of skin AF on top of the classical cardiovascular risk factors has to be studied more extensively. It would also be interesting to study whether or not skin AF could replace the classical cardiovascular risk factors. If so, skin AF will get the chance to develop more potential value to become implemented in clinical practice.

To conclude this paragraph, skin AF assessment is an easily applicable tool that might be valuable in identifying high risk patients for cardiovascular complications or mortality. Clinicians should be aware to translate the skin AF level of an individual patient into a prediction on outcome in this specific individual. The clinical use of skin AF still needs more evidence before implementing this tool in clinical decision making.

Future perspectives

At present, skin AF assessed by the current technique, still poses challenges in interpretation and applicability on an individual patient level, and therefore is (not yet) an instrument which can be advocated for widely clinical use. In general, it should be elucidated which AGEs can be identified as being most representative for vascular damage. However, the most important AGEs might be different in different disease states; for example, in diabetes the compounded effects of oxidation and glycation might lead to more and different toxic substances than the effects of uremia in advanced renal failure. Chances to 'one size fits all' are virtually nonexistent.

We hypothezise that some individuals have skin AF measurements that might be a true representation of the degree of vascular damage by AGEs. Influencing its formation and presence might be of value to help preventing complications. Up to now, we don't exactly know yet to what extent therapies used in daily practice to influence e.g. glucose control, blood pressure, or lipid profile, influence the degree of AGE accumulation, separate from the fact that they possibly decrease the overall rate of AGE accumulation. Many of the known treatment regimens to prevent or delay the further development of micro- and macrovascular complications exert their effects by other (and already reasonably well known) pathways. Therefore, one has to criticize newly introduced measurement techniques or novel biomarkers such as skin AF and Prx4. Their implemention will only be appropriate if they will lead to a consistently different treatment approach. On the one hand, the utility of such novel biomarkers might be useful to predict future cardiovascular events, but, on the other hand, up to now, the additional gains on top of reducing conventional risk factors have been minimal. Nevertheless, it remains important to search for possible new biomarkers as adjuncts to conventional risk factors to provide insight into underlying mechanisms of diseases and new therapies. Biomarkers such as skin AF and Prx4, can become powerful tools when they are clinical applicable and specific enough for an individual subject to draw conclusions on longer term prediction of events, and when they have impact on drug development with prognostic influence on cardiovascular events. The daily clinical use of skin AF will also become more interesting for the individual patient, as soon as there will be clinical evidence of a successful existing

AGE crosslink breaker or specific AGE inhibitor. It offers the opportunity to monitor these therapies to finally accomplish reduction of cardiovascular events. During the past 20 years, research has been done to find efficient AGE breakers without toxicity and applicable in human. Still, there are only positive results in experimental studies: alagebrium, which inhibits intracellular ROS synthesis, significantly inhibits neointimal hyperplasia after carotid injury in diabetic rats. Targeting AGE cross-links with alagebrium (ALT-711) reduced ROS which restored relaxation of arteries in a rat model of type 2 DM (31,32). No clear clinical beneficial effects of alagebrium has been found in human trials yet, but their effects have been studied no longer than 36 weeks, which is probably too short. Whenever AGE breakers will become available in future, skin AF might be one of the most important tools to properly follow up treatment effects of medication administration. Besides the traditional cardiovascular risk factors in highly cardiovascular risk patients, follow-up skin AF measurements might become an additional clinical parameter of the efficacy of the reduction of metabolic burden. If AGE breakers or inhibitors can slow down the formation of AGEs, including elimination of possibly harmfull degradation compounds, the metabolic burden will decrease in favour of metabolic memory. This theory only holds true when skin AF assesses AGEs that are significantly responsible for the deleterious effect on the vascular wall and indeed will be degradated by the AGE breakers or its accumulation in tissue will be slowed down by AGE inhibitors.

We have unpublished data concerning follow-up skin AF assessment in 452 type 2 DM patients (from the already mentioned ZODIAC cohort) after a median follow-up period of 3.2 years. These data showed stable follow-up skin AF levels compared to baseline skin AF levels in 43% of the study population, and 35% had an increase in skin AF levels. Minor positive associations were seen between skin AF level and a longer diabetes duration, lower lipid levels, a lower eGFR, not using an ACE-inhibitor, and/or with previous macrovascular events. These data suggest that in a well-controlled type 2 DM population, a higher degree of continuous oxidative stress based on various factors may play a role in the progression of AGE accumulation, at least when monitored by skin AF. To find more evidence for this hypothesis, follow-up assessment of skin AF should be done after a predetermined period for at least 5 years, in which AGEs could significantly result in either further damage, or improvement under certain strict medical regimen or with AGE breakers.

As it stands now, skin AF measurement has definitively shown potential, but there are uncertainties left yet. As always can be said after a prolonged period of research: more research and specific follow-up studies are warranted to assess the very important issue whether skin AF will earn its place in individual health care with the ultimate goal to contribute to the improvement of life expectancy in high cardiovascular risk populations.

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CHAPTER 10

NEDERLANDSE SAMENVATTING

(DUTCH SUMMARY)

Inleiding

Hart- en vaatziekten (HVZ) behoren met kanker tot de belangrijkste doodsoorzaken in Nederland. In 2010 overleden gemiddeld 58 vrouwen en 51 mannen per dag aan HVZ, waarmee het aandeel van HVZ in de totale sterfte 30% voor vrouwen en 28% voor mannen was. Veelal zijn er risicofactoren aanwezig die bijdragen aan het ontstaan van HVZ. Een aantal bekende risicofactoren voor HVZ zijn roken, hoog cholesterolgehalte, hoge bloeddruk en hoge bloedsuikers. Het is belangrijk deze risicofactoren te onderkennen zodat ze behandeld kunnen worden. Soms blijkt dat het zogenoemde HVZ risicoprofiel waarin de bekende risicofactoren meegewogen worden, niet afdoende is om een juiste inschatting te maken van het HVZ risico. Het is daarom belangrijk onderzoek te blijven doen naar nieuwe methoden en bepalingen die kunnen bijdragen aan een betere identificatie van hoog HVZ risicopatiënten en op die manier van toegevoegde waarde zijn in de bepaling van het HVZ risico. Tevens kan het onderzoek naar deze nieuwe methoden een bijdrage leveren aan het begrijpen van onderliggende ontstaansmechanismen van vaatschade wat uiteindelijk zal resulteren in HVZ.

Hoge bloedglucosewaarden, ook wel hyperglycemie genoemd, spelen evenals oxidatieve stress een belangrijke rol in het ontstaan van vaatschade in hoogrisicopatiënten zoals patiënten met diabetes mellitus (DM) of patiënten met chronische nierziekten. Dit kan uiteindelijk leiden tot HVZ, met als eindstadium nierfalen en overlijden. Oxidatieve stress is een stofwisselingstoestand, waarbij meer dan een normale fysiologische hoeveelheid schadelijke reactieve zuurstofverbindingen (ROS: 'reactive oxygen species') in de cel wordt gevormd of aanwezig is. Dit kan ontstaan door bijvoorbeeld roken, bepaalde medicijnen, overmatig alcoholgebruik, te lange zonblootstelling, obesitas en hyperglycemie. De gevormde reactieve zuurstofverbindingen ontstaan tijdens de verschillende stappen in de stofwisseling van de mitochondriën, de energiecentrale van de cel waar zuurstof nodig is, en beschadigen alle delen van de cel, inclusief eiwitten, vetten en DNA, wat uiteindelijk bijdraagt aan het verouderingsproces. Alle levende wezens die zuurstof nodig hebben voor hun stofwisseling en daarmee de kans lopen op vorming

van schadelijke ROS hebben antioxidanten als beschermingsmechanisme, die de ROS kunnen wegvangen of de beschadigde celbestanddelen kunnen verwijderen of herstellen.

Chronische blootstelling aan hyperglycemische en oxidatieve stress resulteert in cumulatieve en blijvende veranderingen van weefsels, meestal schade. Deze veranderingen kunnen op korte termijn opgetreden zijn of op de lange termijn. De korte termijn veranderingen worden meer bepaald door het 'glycemisch' geheugen, welke weergegeven kan worden door het hemoglobine A1c (HbA1c) gehalte, waarbij een deel van het hemoglobinegehalte in het bloed versuikerd is. Het HbA1c zegt iets over de glucoseregulatie van de voorafgaande 6 tot 8 weken. De veranderingen op de lange termijn kunnen worden weergegeven door de stapeling van versuikerde eiwitten, de zogenoemde 'Advanced Glycation Endproducts' (AGEs). Deze AGEs kunnen binden aan weefsels met een trage omzetting zoals bijvoorbeeld collageen, een eiwitbestanddeel en onderdeel van het bindweefsel in de huid, dat een omzetting heeft van ca. 15 jaar.

In de jaren negentig werd in twee belangrijke studies met diabetes patiënten een relatie gevonden tussen een slechte metabole controle en de ontwikkeling of progressie van complicaties.
Van patiënten met een chronische nierziekte weten we dat zij een verminderde capaciteit hebben om AGEs en hun afbraakproducten uit het bloed te filteren. Hogere concentraties van AGEs in het bloed kunnen een bijdrage leveren aan versnelde stapeling van AGEs in de weefsels. AGEs op zichzelf kunnen de mate van oxidatieve stress ook verhogen, waarmee er een vicieuze cirkel is geïntroduceerd. Wanneer nierpatiënten afhankelijk worden van nierfunctievervangende therapie zoals hemodialyse, kan dit ook weer oxidatieve stress uitlokken en hierbij kunnen ook nog eens de verdedigingsmechanismen tegen oxidatieve stress, de antioxidanten, uitgeput raken.

Bovengenoemde dysbalans betreffende oxidatieve stress status en verhoogde waarden van AGEs leveren hun bijdrage aan HVZ en sterfte op de lange termijn.

Verhoogde waarden van AGEs zouden een bruikbare bepaling van verhoogde glycemische en oxidatieve stress status kunnen weergeven en daarmee de weefselveranderingen op de lange termijn goed kunnen weerspiegelen.

In dit proefschrift wordt een methode beschreven waarbij er door middel van een eenvoudige, niet invasieve meting een inschatting gemaakt kan worden over de hoeveelheid AGEs in de weefsels. Het meetinstrument, de huid autofluorescentiemeter (ook wel AGE reader genoemd), maakt gebruik van de fluorescerende eigenschappen van sommige AGEs. De AGE reader is een draagbaar apparaat dat met behulp van ultraviolet licht de huid kan laten fluoresceren. Een ingebouwde spectrometer analyseert dan de fluorescentie die de huid uitzendt, ook wel huid autofluorescentie genoemd. Berekening door een computerprogramma levert uiteindelijk een bruikbaar getal. In validatiestudies is reeds aangetoond dat huid autofluorescentie een goede maat voor weefsel AGEs is.
Meerdere studies hebben ondertussen aangetoond dat de gemeten huid autofluorescentie bij patiënten met DM en nierinsufficiëntie hoger is dan in een controle groep en dat huid autofluorescentie in deze groepen patiënten geassocieerd is met HVZ en sterfte.

Doelstellingen en uitkomsten van dit proefschrift

Deel I van dit proefschrift beschrijft 4 studies die gedaan zijn in een grote groep type 2 diabetes patiënten uit de eerstelijns gezondheidszorg (huisartsenpraktijken) in Zwolle. De patiënten waren allen afkomstig uit het ZODIAC project (Zwolle Outpatient Diabetes project Integrating Available Care), wat in 1998 startte en tot op heden voortduurt. In het kader van dit 'transmurale zorg' project worden deze patiënten jaarlijks door een diabetesverpleegkundige gescreend op complicaties, waarna de huisarts aan de hand van deze bevindingen een behandeladvies krijgt van een internist. Tussen 2001 en 2002 werden er bij 973 type 2 diabetes patiënten huid autofluorescentie metingen gedaan. Binnen deze groep werd tussen 2004 en 2005 een 2^{e} huid autofluorescentie meting gedaan bij 452 patiënten.

In hoofdstuk 2 werd de mogelijk voorspellende waarde van huid autofluorescentie ten aanzien van de ontwikkeling van microvasculaire complicaties onderzocht in de hierboven beschreven groep type 2 diabetes patiënten. Deze studie toonde aan dat in deze goed gereguleerde type 2 diabetes patiënten huid autofluorescentie een belangrijke voorspellende waarde heeft voor de ontwikkeling van microvasculaire complicaties in het algemeen vergeleken met de traditionele risicofactoren. Daarnaast bleek het ook een onafhankelijke voorspeller voor de ontwikkeling van de afzonderlijke microvasculaire complicaties neuropathie (zenuwschade) en albuminurie (de aanwezigheid van albumine [= een eiwit] in de urine dat wijst op nierschade) te zijn.

Hoofdstuk 3 bestudeerde de voorspellende waarde van glycemische controle voor de veranderingen in weefselaccumulatie van AGEs in de hierboven genoemde groep diabetes patiënten van de ZODIAC studie, waarin twee huid autofluorescentie metingen waren verricht. Hypothetisch gezien zou de stapeling van AGEs in type 2 diabetes patiënten voorspeld kunnen worden door het HbA1c beloop gedurende een bepaalde periode van jaren voorafgaand aan de huid autofluorescentie meting.

Van verschillende soorten HbA1c bepalingen (HbA1c variantie, gemiddelde HbA1c, maximum HbA1c en aanvangs-HbA1c) werd onderzocht of ze geassocieerd waren met de snelheid van de aanmaak en stapeling van AGEs over een bepaalde tijd. In deze studie werd rekening gehouden met variabelen die eveneens van invloed konden zijn op de aanmaak en stapeling van AGEs: beginwaarde van huid autofluorescentie, leeftijd, diabetesduur, nierfunctie en roken. De resultaten van deze studie toonden aan dat veranderingen in huid autofluorescentie niet goed konden worden voorspeld door de verschillende soorten HbA1c bepalingen.

In hoofdstuk 4 werd de antioxidant peroxiredoxine 4 (Prx4) bestudeerd. Zoals in het eerste deel van de samenvatting werd uitgelegd, hebben alle aërobe organismen beschermingsmechanismen tegen oxidatieve stress: de antioxidanten die de schadelijke reactieve zuurstofverbindingen (ROS) kunnen wegvangen of de beschadigde celbestanddelen kunnen verwijderen of herstellen. De peroxiredoxines zijn enzymen waarvan er 6 isovormen bestaan, en spelen een rol in de afbraak van de ROS, waarvan Prx4 de enige isovorm is die in het bloed meetbaar is. Er werd onderzocht of Prx4 geassocieerd zou kunnen zijn met sterfte ten gevolge van HVZ

én sterfte door alle oorzaken in een type 2 DM populatie uit het eerder genoemde ZODIAC cohort. Deze studie toonde voor het eerst aan dat Prx4 een voorspeller bleek voor zowel sterfte ten gevolge van HVZ als voor sterfte door alle oorzaken. Er werd eveneens gesuggereerd dat het een potentieel nieuwe ziekte-indicator voor HVZ zou kunnen worden.
De levensverwachting van type 2 diabetes patiënten in de genoemde ZODIAC studie werd in hoofdstuk 5 bestudeerd. Dit werd vergeleken met die van de algemene Nederlandse bevolking. Er bleek in deze studie sprake te zijn van een normale levensverwachting in deze groep type 2 diabetes patiënten ten opzichte van de algemene Nederlandse bevolking. Tevens waren zowel een voorgeschiedenis met HVZ als albuminurie nog wel een voorspellende waarde voor een verminderde levensverwachting, wat we uit andere studies ook weten. Door een goede diabeteszorg te leveren en het behalen van de streefwaarden wat betreft o.a. glucoseregulatie, bloeddruk en cholesterolprofiel zou er wellicht een gunstiger metabool profiel kunnen ontstaan, wat mede zou kunnen bijdragen aan de verbeterde levensverwachting over de afgelopen jaren.

Deel II van dit proefschrift omvat een drietal artikelen die betrekking hebben op de stapeling van AGEs en huid autofluorescentie in patiënten met chronisch nierfalen en hemodialyse patiënten.
Hoofdstuk 6 is een overzichtsartikel over AGEs, huid autofluorescentie en nierfunctie. Hierin wordt benadrukt dat patiënten met chronisch nierfalen een verminderde nieruitscheiding van AGEs en hun afbraakproducten uit het bloed hebben. Er wordt eveneens beschreven dat er meer oxidatieve stress in patiënten met chronisch nierfalen en hemodialyse patiënten is, wat op zijn beurt weer een extra aanleiding geeft tot vorming en stapeling van AGEs. Daarnaast staan er in hoofdstuk 6 originele data vermeld wat betreft huid autofluorescentie in relatie staande tot nierfunctie bij de type 2 diabetes patiënten van de in Deel I genoemde ZODIAC studie. In tegenstelling tot wat er verwacht werd, bleek huid autofluorescentie in deze groep niet een duidelijke correlatie met de nierfunctie te tonen.
De toepasbaarheid wat betreft de meting van huid autofluorescentie als risicobepaling voor diabetische nefropathie (nierziekte als complicatie van DM) als voor HVZ in

niet-diabetisch nierfalen, milde nierfunctiestoornissen en niertransplantatie wordt besproken in hoofdstuk 7. Hierin wordt genoemd dat AGEs een belangrijke rol spelen in zowel de ontwikkeling van diabetische nefropathie als in niet-diabetisch nierfalen. In dit artikel wordt eveneens een kritische houding aangenomen ten opzichte van de medicamenteuze interventies om de vorming en stapeling van AGEs tegen te gaan. Behandeling met deze middelen vindt enkel in studieverband plaats en is tot op heden nog niet klinisch toepasbaar.
In hoofdstuk 8 werd de voorspellende waarde van huid autofluorescentie voor sterfte ten gevolge van HVZ als voor sterfte door alle oorzaken in een groep van 105 hemodialysepatiënten onderzocht. Naast de traditionele risicofactoren bij hemodialysepatiënten, bleek huid autofluorescentie enkel een voorspeller voor sterfte door alle oorzaken te zijn.

Samenvattend beschrijft dit proefschrift verschillende factoren die de ontwikkeling van HVZ risico in patiënten met type 2 DM en patiënten met chronisch nierfalen of eindstadium nierfalen mede bepalen. In deze hoogrisicopatiënten hebben we aangetoond dat huid autofluorescentie een voorspeller is voor het optreden van ziektegerelateerde complicaties en sterfte. Eveneens werd in een groep type 2 diabetes patiënten de associatie tussen een potentieel nieuwe ziekte-indicator Prx4 en sterfte bestudeerd én er was aandacht voor voorspellers voor sterfte en levensverwachting in deze populatie vergeleken met de algemene Nederlandse bevolking.

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